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CLINICAL, CYTOGENETIC AND MOLECULAR CHARACTERIZATION OF SEVEN PATIENTS WITH DELETIONS OF CHROMOSOME 22q13.3

BY

NANCY J. NESSLINGER

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

Department of Genetics

Edmonton, Alberta
Fall, 1993

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This thesis is dedicated to the memory of my grandfather
Alois Nesslinger

ABSTRACT

A molecular study of seven patients who have chromosome 22q13.3 deletions as revealed by high resolution cytogenetic analysis was undertaken. Clinical evaluation of the patients revealed a common phenotype that included generalized developmental delay, normal or accelerated growth, hypotonia, severe delays in expressive speech and mild facial dysmorphic features. RFLP and dosage analysis using a series of genetically mapped probes showed that the proximal breakpoints of the deletions varied over approximately 13.8 cM, between loci D22S92 and D22S94. The most distally mapped probe, ARSA, was shown by densitometry and fluorescent in situ hybridization to be deleted in all seven patients. Therefore, the smallest region of overlap (critical region) extends between locus D22S94 and a region distal to ARSA, a distance of more than 25.5 cM. Using the D22S95 probe, pulsed field gel electrophoresis has demonstrated the presence of possible rearrangement bands in two patients. Yeast artificial chromosomes were used to try and span the region that contains the two proximal breakpoints, defined by the loci D22S95 and D22S64. Finally, several clones that may identify genes were localized to the critical region defined by the seven patients. This study is the first extensive characterization of a new deletion syndrome.

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ABBREVIATIONS

HPRT	Hypoxanthine phosphoribosyltransferase
REM	Rapid eye movement
RFLP	Restriction fragment length polymorphism
PFGE	Pulsed field gel electrophoresis
YAC	Yeast artificial chromosome
FISH	Fluorescent *in situ* hybridization
D-MEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
EDTA	Disodium ethylene diamine tetraacetate
PMSF	Phenazine methosulfate
LB	Luria-Bertani medium
PEG	Polyethylene glycol
SDS	Sodium dodecyl sulfate
TNE	10 mM Tris pH 8.0, 10 mM NaCl, 2 mM EDTA
UV	Ultraviolet
SSC	0.15 M NaCl, 0.015 M Nacitrate
CHEF	Clamped homogenous electric field
kb	Kilobase
FB, QM, CH, CB, AJ, NS, JT	patient designations

INTRODUCTION

Review of chromosome 22 and its anomalies

Human chromosome 22 is the second smallest autosome, containing approximately 52 Mb of DNA or 1.8% of the human genome. It is an acrocentric chromosome with a p-arm polymorphic in size due to variable amounts of heterochromatin. Ribosomal RNA genes are the only active genes known to be located in the p-arm and are visualized by NOR staining (Kaplan et al, 1987). The q arm contains primarily euchromatin. At the 400 band stage, the q arm has two light G-bands (q11 and q13) separated by one dark G-band (q12), each of which are further subdivided at the 850 band stage. Only approximately 5% of the total predicted number of genes (1000-2000) on chromosome 22q have been identified. Data has shown that chromosome 22 undergoes more recombination than is expected based on physical size. The genetic map of approximately 97 cM exceeds the calculated physical map by more than 60%, indicating a high frequency of recombination (Rouleau et al, 1989). This has been confirmed on a long-range restriction map in which 1 cM equals approximately 260-320 kb in the region between the immunoglobulin λ locus (22q11) and the Ewing sarcoma breakpoint (22q12) (McDermid et al, in press).

Despite its small size, chromosome 22 is associated with many acquired and congenital chromosome anomalies. The acquired abnormalities involve a number of specific cancer-related translocations or deletions. These include the t(9;22)(q34.1;q11.2) translocation involved in chronic myelogenous leukemia which

produces a truncated 22 known as the Philadelphia chromosome (Nowell and Hungerford, 1960; Rowley, 1973), the t(8;22)(q24;q11) variant translocation associated with Burkitt's lymphoma (Berger et al, 1979), and the t(11;22)(q24;q12) translocation involved in Ewing's sarcoma (Aurias et al, 1983), a small round cell neuroectodermal tumor occuring in the trunk or extremities (Selleri et al, 1991). Meningioma, a common tumor arising from the arachnoidal cells surrounding the brain, often shows deletions of 22q11-22q12 (Deprez et al, 1991) or the entire chromosome totally lost (Zang et al, 1982). This tumor is a frequent cause of seizures and is usually considered to be benign, however, it may become malignant leading to progressive neurological disability and death (Seizinger et al, 1987). Bilateral acoustic neurofibromatosis (BANF) or neurofibromatosis type 2 (NF2) is an autosomal dominant disorder also associated with chromosome 22 deletions. The hallmark of the disease is the development of bilateral acoustic neuromas, which are Schwann cell derived tumors (Martuza and Eldridge, 1988). In addition there is a predisposition to central nervous system tumors including meningioma, ependymomas, spinal neurofibromas and gliomas (Rouleau et al, 1990). The use of family studies and tumor deletion mapping has narrowed down the location of NF2 to within the chromosomal band 22q12 (Rouleau et al, 1990; Wolff et al, 1992). A gene from the NF2 region involved in nonoverlapping and interstitial deletions in four independent NF2 patients was recently identified (Troffater et al, 1993). The candidate gene encodes a novel protein related to the moesin-ezrin-radixin family of cytoskeleton-associated proteins which may represent a new class of

tumor suppressor whose function is mediated by interactions with the cytoskeleton (Troffater et al, 1993). Finally, some pediatric brain tumors, specifically rhabdoid or atypical teratoid tumors, demonstrate monosomy 22 as the only cytogenetic abnormality (Biegel et al, 1990).

There are several important congenital anomalies involving chromosome 22. The balanced t(11;22) translocation is the most common non-Robertsonian translocation in humans and is often associated with reproductive failure due to production of unbalanced gametes (Fraccaro et al, 1980). Cat eye syndrome, which is characterized by ocular coloboma, anal and cardiac defects, and mental retardation, is usually associated with a bisatellited supernumerary chromosome which was shown to be derived from chromosome 22 (McDermid et al, 1986). DiGeorge syndrome (DGS), is a developmental field defect of the third and fourth pharyngeal pouches. It is characterized by aplasia or hypoplasia of the thymus and parathyroid glands and conotruncal cardiac defects (de la Chapelle et al, 1981). DGS has been shown to be associated with a deletion or microdeletion of chromosome 22q11 (Driscoll et al, 1992a; Scambler et al, 1991). A similar microdeletion is also associated with velo-cardio-facial syndrome (VCFS), which is characterized by cleft palate, structural cardiac anomalies, learning disabilities and a typical facial appearance (Driscoll et al, 1992b). In addition, 5 of 9 families studied with recurrent outflow-tract heart defects have a deletion in chromosome 22q11 detected by the same probes that are deleted in most DGS and VCFS patients (Wilson et al, 1992)

Although chromosome abnormalities involving 22q11-12 have been well studied, little is known about chromosome abnormalities of 22q13, the most distal band. There have been as few as six reports of patients described as having deletions of the distal portion of chromosome 22. Watt et al (1985) reported a family segregating a pericentric inversion of chromosome 22 in which the proband was found to be microcephalic, with mental retardation and no speech. Cytogenetic analysis revealed a 46,XY,del(22)(q12) karyotype. A male with a 46,XY,del(22)(q13.31) karyotype and a severe phenotype typical of Goldenhar complex was reported by Herman et al (1985). The phenotype included macrocephaly, preauricular tags, seizures and developmental delay. Kirschenbaum et al (1988) described a 46,XY,del(22)(q12) male who showed developmental and speech delay. Romain et al (1990) described a patient with a karyotype of 46,XX,del(22)(q13.1) who demonstrated developmental delay, hypotonia and no speech. A 46,XX,del(22)(q13.31) female was described by Narahara et al (1992) with hypotonia, developmental delay, and mildly dysmorphic features. Finally, Phelan et al (1992) reported a male with a terminal deletion of 22q13.31 who demonstrated developmental delay, hypotonia, and multiple minor anomalies. A more complete description of each case is provided in Table 1.

The other major category of chromosome 22q13 rearrangements is the ring (22) chromosome in which there is usually a deletion of pter-p11 and q13-qter, followed by joining of the two ends. There have been many patients described with a ring(22). The most common findings include hypotonia, mild to

Table 1- Clinical comparison of 6 published cases with deletions of the distal portion of chromosome 22

	Case					
	1	2	3	4	5	6
Perinatal						
Appropriate for gestational age	?	+	?	+	+	+
Term gestation	?	+	?	+	+	+
Cesarian section	?	+	?	-	-	-
Neurological Abnormalties						
Hypotonia	-	-	-	+	+	+
Developmental delay	+	+	+	+	+	+
Delay of gross motor milestones	+	+	+	+	+	+
Delays or absence of expressive speech	+	?*	+	+	?*	+
Macrocephaly	-	+	-	-	-	-
Mild dilatation of cerebral ventricles	-	+	-	-	-	-
Seizures	-	+	+	-	-	-
Dysmorphic features						
Dolicocephaly	-	+	-	-	-	+
Ptosis	-	+	-	-	-	+
Epicanthal folds	+	+	+	+	-	+
Dysplastic ears	-	+	-	+	+	+
Highly arched palate	-	-	-	-	+	-
Simian crease	-	+	-	-	-	+
Syndactyly	+	-	-	-	-	+
Tall stature	-	+	-	+	-	-

+- feature present - feature absent ?- information not provided
?*- patients too young to ascertain

Isolated findings limited to a single patient:
Case 1; lordosis, microcephaly, deep set eyes
Case 2; facial asymmetry, bilateral dermoids, bilateral hydronephrosis hydrocephalus, possible lissencephaly
Case 3; Broad fingers and thumbs
Case 4; clubbed feet
Case 5; upslanted palpebral fissures, micrognanthia

Published cases
Case 1. Watt et al, 1985
Case 2. Herman et al, 1985
Case 3. Kirschenbaum et al, 1988
Case 4. Romain et al, 1990
Case 5. Narahara et al, 1992
Case 6. Phelan et al, 1992

severe mental retardation, developmental delay, and microcephaly. However the phenotype of ring(22) cases is extremely variable. There have been reports of macrocephaly (Christodoulou et al, 1992) and cases where there is no mental retardation (Brookfield et al, 1976). The phenotype of ring(22) cases is generally so variable that no specific syndrome has been assigned to the ring(22) cases.

Ring chromosomes are not condusive to study because they are extremely unstable. Due to this mitotic instability, various ring derivatives may be formed or the ring may be lost during cell division. Most of the ring(22) cases reported demonstrated some degree of mosaicism. In some cells the ring may have been lost, rendering the cell monosomic for that chromosome. In other cells a dicentric, double ring is present, formed by a sister chromatid exchange within the monocentric ring chromosome. In some cells, a normal karyotype is present. The majority of cells, however, show a single ring chromosome with no additional chromosomal rearrangements.

There are several theoretical explanations to account for the phenotypic variability seen in ring(22) chromosome cases. First, the formation of a ring is thought to result from breakages of both ends segments of the chromosome, followed by the broken ends rejoining to create a continuous ring (Kosztolayni, 1987). Presumably, some genetic material is lost from both ends. Since loss of the short arm of chromosome 22 seems to be compatible with normal development (Nielson et al, 1974), the severity of the phenotype probably reflects the amount of genetic material lost from the q arm. Thus, the variability may reflect the variety of breakpoints in the q arm and

the amount of chromosomal material deleted. However, there may be some instances in which a ring is formed by telomere association and significant deletion of chromosomal material is not involved in the formation of the ring (Kosztolayni et al, 1991). If this is the case, the variation in stability of the ring in different tissues at different developmental times may be responsible for the phenotype. This cannot be studied therefore to study deletions of chromosome 22q13 it is best to consider only isolated 22q13 deletions.

Proposed models of aneuploidy

In studying an aneuploidy syndrome, there is a need to consider the correlation of phenotype and genotype. Several models have been proposed to explain the effect of aneuploidy on the phenotype of the aneuploid individual. That is, how does a particular state of chromosome imbalance produce a specific set of abnormalities. The classical definition of aneuploidy refers either to a whole chromosome duplication, such as trisomy 21, or a whole chromosome deletion, such as monosomy 21. In this thesis, the term aneuploidy will be expanded to include partial chromosome duplications or deletions. There are two general models that have been proposed. The first, supported by Burton Shapiro (Shapiro, 1970), is one of amplified developmental instability in which a generalized disruption of evolved homeostasis is responsible for the aneuploid phenotype. The second, proposed by Charles Epstein (Epstein et al, 1986), suggests that the complex phenotypic effects of an aneuploid state should be able to be reduced to the imbalance of a specific genetic locus or set of loci.

Shapiro's work centers on how a triplicated chromosome 21 could result in the Down syndrome phenotype. It was previously suggested that qualitative gene content was not as important as quantitative gene imbalance and that the extra chromosome may act as a source of diffuse disturbance (Hall, 1965). This idea led Shapiro to hypothesize that the extra chromosome in Down syndrome results in a generalized decrease in developmental homeostasis or canalization (Shapiro, 1970). The balancing process in physiology that maintains a steady state is called homeostasis. It has been suggested that the genotype is buffered such that evolved developmental pathways are stabilized against inevitable genetic and environmental disturbances and are returned to the standard track. This buffering and channeling in development has been termed canalization (Shapiro, 1983). Therefore we can view canalization as developmental homeostasis, the mechanism that allows development to proceed towards a standard mean.

The physical diagnosis of Down syndrome is an impressionistic one based on the sum of numerous abnormalities; none of which, except the extra chromosomal material, is specific or unique to Down syndrome. That is, all reported findings in Down synrome occur isolated in the general population. In addition, there is an overlap of findings among autosomal trisomy syndromes, such that the overlap of anomalies among trisomies and the general population is as dramatic if not more dramatic than the clinical differences (Shapiro, 1983). However, if one actually compares the major characteristics (present in at least 50% of cases) of trisomy 13, 18 and 21, it is obvious that these are distinct, recognizable syndromes. The

following is a brief summary of the major characteristics found in trisomy 13, 18 and 21 according to Smith's Recognizable Patterns of Human Malformation (Jones, 1988). Trisomy 13 is characterized by holoprosencephaly, severe mental retardation, deafness, moderate microcephaly, cleft lip and/or palate, polydactyly, narrow hyperconvex fingernails, cardiac malformations and scalp and skin defects. Trisomy 18 is characterized by growth deficiency, mental retardation, clenched hands, low arch dermal ridge patterning on fingertips, a short sternum, and cardiac malformations. Trisomy 21 or Down syndrome is characterized by hypotonia, hyperflexibility of joints, slanted palpebral fissures, a flat face, mental retardation, and dysplasia of the pelvis. The major cause of early mortality is congenital heart defects, present in 45% of cases. Thus, when considering the most common findings in each of these trisomies, it is apparent that they are not that similar and that they are clearly distinguishable on physical examination.

There is also marked phenotypic variability in Down syndrome. In fact, the Down syndrome population is more variable than the general population for many if not most metric traits (Shapiro, 1970). Thus, in Down syndrome, the extra chromosome is thought to cause a generalized disruption of evolved genetic balance. This disruption results in decreased developmental and physiological buffering against genetic and environmental forces, which in turn will lead to decreased developmental and physiological homeostasis or canalization (Shapiro, 1983) and increased variances. Therefore, individuals with a chromosomal imbalance are more likely to have defects than the general population.

Normal traits that possess large environmental contributions to variability, in general, have been shown to be less stable than those traits with small environmental variance (Shapiro, 1975). If Shapiro's hypothesis is correct you would expect to observe that the more unstable pathways will be most seriously reflected in the Down syndrome phenotype (Shapiro, 1970). In other words, the instability is amplified in a trisomy due to a disruption that leads to decreased buffering. This model predicts that generalized diminished buffering will result in i) increased variance of metric traits (a measure of decreased developmental homeostasis), ii) amplified instability of developmental pathways, iii) reduced precision of physiologic homeostatic controls, and iv) increased susceptibility to genetic and environmental trauma (Shapiro, 1983).

To test the model Shapiro looked at many metric traits, including both those with little environmental contributions to variability and those with higher levels of environmental contributions, as well as several physiological measurements. Initially Shapiro analyzed dentition, comparing the Down syndrome population with the general population. Hypodontia, the condition where one or more teeth are missing, was approximately 4-5 times greater in Down syndrome than in the general population. However, the distribution of specific missing teeth was similar. This therefore may suggest an amplification of susceptibility rather than specific target defects (Shaprio, 1970). Several reports also demonstrated that in Down syndrome teeth are microdontic, such that all teeth in Down syndrome patients were smaller than in normal individuals. This led to the conclusion that individual tooth types exhibit a wider

variation in size in Down syndrome compared to the general population (Shapiro, 1970). However, if, on average, all teeth in Down syndrome are smaller, it does not necessarily indicate greater variance, it only indicates a shift in the mean. Shapiro also looked at tooth eruption. Tooth eruption normally occurs at definite times in definite sequence, however radical differences are seen in the general population. In Down syndrome, eruption is clearly delayed and eruption usually proceeds in an abnormal sequence compared to normal. All of these differences were statistically significant. Overall, the type of tooth missing or abnormal in Down syndrome is parallel to that in the general population, but those teeth that are less stable in their development in the general population are more frequently and more severely affected in Down syndrome (Shapiro, 1970).

Shapiro also looked at palatometric (involving the palate) and dermatoglyphic traits in normal and Down syndrome individuals. In studying the palatometric measurements, Shapiro considered the environmental contribution to palatal height, width and length by measuring these dimensions in monozygotic and dizygotic twins as well as Down syndrome and normal individuals. By comparing the variance in dizygotic twins and monozygotic twins, it is possible to estimate genetic contribution to the variability of a trait. In the sample studied, it was found that variation in palatal length was due mainly to the environment, palatal width had slight genetic contribution and palatal height had the greatest amount of genetic contribution of the three traits. Since environmental variance is an indication of the degree of developmental homeostasis, the greater

the environmental contribution, the less stable the pathway. Therefore palatal length is the least stable trait developmentally. When the Down syndrome individuals were compared to the normal individuals, Shapiro found that palatal length was reduced so greatly in Down syndrome patients that Down syndrome patients could be picked out by this trait alone. The dimension having the next highest relative contribution of environment, palatal width, was second most deviant in the Down syndrome sample, who all fell about 2 standard deviations below the normal mean. Nearly all (95%) of Down syndrome subjects fell within 2 standard deviations of the control mean for palatal height, the dimension having relatively the smallest environmental contribution to variability. Thus, of the three dimensions examined, the one most subject to the environment during development is the most abnormal in Down syndrome (Shapiro, 1975).

When considering dermatoglyphics, total fingertip ridge count is thought to depend on nearly perfectly additive genes and environmental factors play only a small part in production of the phenotype. This is considered the most stable metrical character in man (Shapiro, 1975). Total ridge counts in Down syndrome do show a statistically significant difference compared to normal; however, the deviations are not that dramatic. Palmar *atd* angle is influenced strongly by the environment at early developmental stages. When comparing this trait in Down syndrome and normal individuals, a very substantial and consistent deviation from the normal population is seen in Down syndrome. Thus again the developmentally less stable trait is more deviant in Down syndrome (Shapiro, 1975).

In all of these examples, there is not so much an increase in variability in the Down syndrome population as a shift in the mean, when considering metric traits. Shapiro's model predicts only that decreased cannalization leads to an increased variance of metric traits (Shapiro, 1983), and that the increased variance should fall around the normal mean. However, what is seen with most metric traits in the Down syndrome population is a decrease in the mean when compared to the general population and not an increase in the variance about the mean. Shapiro suggests that the population mean of a trait be viewed as an optimum achieved during a species' history and that a disruption of the basis for this optimum would tend to be unidirectional (Shapiro, 1983). That is, chromosome imbalance would affect development such that developmental thresholds may not be attained, leading to a general deficit in characteristics. Thus, an increased susceptibility to genetic and environmental disturbances because of decreased buffering would probably affect development adversely. What remains is the fact that many metric traits in Down syndrome show a decrease in the mean rather than an increase in variability around the mean.

Shapiro also considered some physiological traits. Fundamental physiological homeostatic mechanisms are crucial for survival, so they must be buffered accurately. The model presented by Shapiro predicts that individuals with Down syndrome, when faced with a physiological challenge, will respond with a higher peak and/or a delay in return to baseline (Shapiro, 1983). To support this, Shapiro found that glucose regulation is less well buffered in Down syndrome individuals and that sensory evoked electroencephalographic (EEG)

responses are increased when presented with a stimulus but adaptation to the stimulus is decreased (Shaipro, 1983).

With these examples, Shapiro was able to demonstrate how the Down syndrome phenotype may be due to amplified developmental instability. The problem with this model arises when one considers that Down syndrome is a distinct clinical entity. As already indicated, there are similarities between the autosomal trisomies, however there are significant phenotypic differences that allow diagnosis on physical examination. Shapiro acknowledges that dosage effects of extra or diminished gene loci on the aneuploid chromosome do have an effect. The altered dose of these gene products probably affect the phenotype in ways different from loci on other chromosomes (Shapiro, 1989). The primary dosage differences probably also lead to dosage effects of gene products on other chromosomes and these could have effects that would not necessarily be predicted from the aneuploid loci (Shapiro, 1989).

Like Shapiro, Charles Epstein's work centers on Down syndrome. His views regarding how an aneuploidy can lead to a specific phenotype stem from his work attempting to define a Down syndrome region. Epstein believes that implicit in all attempts to find a "Down syndrome region" is the assumption that the phenotypic manifestations of an aneuploid syndrome can be assigned to a specific region or regions of the chromosomal imbalance (Epstein, 1990). To assess the validity of this assumption, Epstein undertook an analysis of the clinical and cytogenetic descriptions of a large number and variety of cases of aneuploidy, both deletions and

duplications. From this analysis he was able to propose two principles concerning the pathogenesis of aneuploid phenotypes.

The first principle states that individual phenotypic anomalies or features can often be assigned or mapped to specific regions of the genome and conversely, the component manifestations of individual aneuploid states can often be added together to generate the phenotypes of combined aneuploidies (Epstein, 1986). This principle is based on the comparison of phenotypes resulting from simultaneous imbalance of two chromosomes, or two chromosome segments, or from progressively larger degrees of imbalance of a single chromosome (Epstein, 1990). The most convincing support for this principle comes when examining the effects of unbalancing regions of increasing length from the same chromosome, the basis for phenotypic mapping. When considering several such cases involving duplication of 5q, 7q, 9q, 10q and deletions of 11q, 4q, and 22pter-q11, Epstein was able to conclude that phenotypic features of short aneuploid segments are generally included among the features manifested by aneuploidy of longer segments which include them. In addition, it is possible to identify certain features with aneuploidy for specific chromosome segments (Epstein, 1986).

The best example of phenotypic mapping is provided by Down syndrome. Down syndrome is generally identified with trisomy 21, but in order to identify genes that could play a role in the pathogenesis of the condition, definition of minimal regions on chromosome 21 involved in producing particular features of the phenotype is essential. This can be accomplished by analyzing genotype-phenotype correlations in rare patients with partial

trisomy 21. The features of Down syndrome that these patients possess allow more precise localization of the region responsible for their phenotype when considering their particular chromosome rearrangement. In a recent report (Delabar et al, 1993), ten patients with partial trisomy 21 for different segments of chromosome 21 were analyzed, leading to the molecular mapping of 24 Down syndrome features on chromosome 21. Previous karyotypic studies have indicated that trisomy of chromosome 21q22 alone was sufficient to cause Down syndrome (Mattei et al, 1981). This has been confirmed by the Delabar (1993) study. They found that nineteen Down syndrome features could be assigned to just 2 regions of chromosome 21. Short stature, joint hyperlaxity, hypotonia, major contributions to mental retardation and 9 anomalies of the face, hand and foot were all localized to 21q22.2 or proximal 21q22.3, a region spanning 0.4-3 Mb around D21S55. This region was defined as the Down syndrome chromosome region (DCR). Six facial and dermatoglyphic anomalies were assigned to a 6 Mb region located on 21q22.2 and part of 21q22.3, a region that included the DCR (Delabar et al, 1993). They suggest that the complex phenotype of Down syndrome may in large part be the result of overdosage of only one or a few genes within the DCR (Delabar et al, 1993). However, this analysis was based on a small number of cases and expression of Down syndrome is extremely variable, and these facts need to be remembered when interpreting their results.

There are problems associated with reconstructing phenotypes from sub-phenotypes. There is a certain amount of "noise" which extends beyond that expected from variability of expression. When

deduced sub-phenotypes are combined, certain phenotypic manifestations may disappear and new ones, not associated with either sub-phenotype, may appear (Epstein, 1986). This is not surprising because there are new interactions that would not be possible when just one region is unbalanced. What is surprising is that obliteration or appearance of new phenotypic traits does not occur more frequently. From this Epstein has inferred that those features that do remain unaltered result from imbalance of loci that have a strong and deterministic effects on development and that these are the loci that should ultimately be readily identifiable (Epstein, 1990).

The second principle proposed by Epstein states that different aneuploid phenotypes, while often variable in expression and frequently overlapping in phenotype, are each specific and differentiable from one another (Epstein, 1986). This principle asserts that there is a specific and deterministic relationship between imbalance of a particular region of the genome and the syndrome of defects that occurs as a result of this imbalance. This principle argues against the suggestion that the manifestations of aneuploidy are the result of non-specific disturbance of general chromosome imbalance (Epstein, 1990). Epstein suggests that this proposition has been advanced for two reasons. The first is that there is a considerable degree of variability in aneuploid phenotypes and any two individuals with the same aneuploid state do not necessarily have identical phenotypic abnormalities. Phenotypic variability should not be a surprise. Even when one looks at an autosomal dominant disorder, such as Marfan syndrome, widely divergent

manifestations are seen, even within the same family. To account for this variability it must be remembered that, except for identical twins, individuals have different genetic backgrounds. It has been shown experimentally in animals that differences in genetic background can influence the ultimate phenotype (Epstein, 1986). In addition, even identical twins may not have identical phenotpes. There are stochastic and environmental factors that introduce small elements of randomness into the operation of a developmental pathway (Epstein, 1990).

The second reason the non-specificity theory arises is because many aneuploid syndromes share similar phenotypic abnormalities. Mental retardation and growth impairment are nearly universal features when significant aneuploidy is present. However, despite the overlaps it is still possible to discriminate different and often quite closely related aneuploid phenotypes from one another. Epstein believes that the overlapping phenotypes can be explained if we realize that many different enzymatic defects scattered throughout the genome can have quite similar phenotypic effects (Epstein, 1988). Although Epstein acknowledges that amplified developmental instability may affect the stability of certain developmental processes, he does not believe that this mechanism could produce most of the phenotypic abnormalities of a syndrome (Epstein, 1986). He argues that it would not be possible to discriminate among the different aneuploid syndromes if that were the case (Epstein, 1989).

According to Epstein, there is only one thing that can explain aneuploid phenotypes; the cumulative effect of the imbalance of the

individual genes located on the unbalanced chromosome or chromosome region. There are numerous mechanistic precedents demonstrating how small changes in gene dosage could or may have major effects on cellular function and gene expression (Epstein, 1986). Thus, Epstein believes it will ultimately be possible to explain an aneuploid phenotype from a knowledge of the functions of the individual genes present in the unbalanced region. It will likely not be possible to explain each phenotypic feature by imbalance of a single locus. For some features, imbalance of two or several genes will combine to produce the observed outcome (Epstein, 1988).

It is reasonable to imagine that certain aneuploid states may affect the stability of certain developmental processes, however there is no evidence to prove that aneupoidy, independent of which chromosome is involved, leads to generalized disturbance of developmental homeostasis affecting many if not all morphogenetic processes. This model is attractive in that it may possibly explain some of the non-specific features of Down syndrome, however it cannot account for the fact that one can distinguish aneuploid phenotypes from one another. In addition, Shapiro has never applied his model of amplified developmental instability to any other aneuploid syndrome besides Down syndrome, and he did not consider many of the traits that are diagnostic of Down syndrome. Thus, it is not clear if his model could account for other deletion or duplication syndromes. It is much easier to imagine that aneuploid phenotypes are the result of the cumulative effect of imbalance of the individual genes located on the unbalanced chromosome or chromosome region such as Epstein suggests.

Overall, it is likely that a combination of the two theories contribute to the effect of aneuploidies on the phenotype. Some traits, such as the mental and growth retardation seen in almost all aneuploid syndromes, may be due to amplified developmental instability. However, other traits that are characteristic of a particular syndrome such a specific organ defects, are more likely to be due to specific genes. Therefore it makes sense to map syndromes, determine their critical region and look for genes responsible for the phenotype of the syndrome.

The first example of a probable specific gene dose effect as the cause of a human disease has been identified in Charcot-Marie-Tooth (CMT) disease. CMT is the most common inherited peripheral neuropathy in humans, characterized by progressive muscular atrophy that primarily affects the distal muscles. There are two types of CMT: CMT type 1 is characterized by uniformly decreased nerve conduction velocities and CMT type 2 has normal or near normal nerve conduction velocities (Patel, 1993). CMT type 1 has been the most extensively studied and one locus (CMT1A) thought to cause the disease has been mapped to chromosome 17p11.2-p12 (Vance et al, 1989). CMT1A was demonstrated to be completely linked and associated with a large submicroscopic duplication in 17p11.2-p12 (Lupski et al, 1991), representing an unprecedented mutational mechanism for an inherited disease. Further studies indicated that the duplication is a tandem repeat of 1.5 Mb, suggesting that *de novo* CMT1A arises from the unequal crossing over between non-sister chromatids of homologous chromosomes due to misalignment during meiosis I of the repeat sequences found

at the breakpoint of the duplication (Pentao et al, 1992). The reciprocal deletion product of this exchange was shown to be associated with hereditary neuropathy with liability to pressure palsies (HNPP), an autosomal dominant disorder producing episodic, recurrent demyelinating neuropathy (Chance et al, 1993).

Several models were proposed to explain how the DNA duplication might affect expression of a candidate CMT1A gene(s) and result in the CMT1A phenotype. The models included 1) overexpression of one or more genes in the duplicated region or a gene dosage effect, 2) interruption of a candidate gene at the duplication junction, 3) occurrence of a stable dominant mutation in one of the duplicated genes, and 4) a change in the physical location of the gene(s) within the duplicated region causing a position effect (Lupski et al, 1992). To investigate the gene duplication model, a partial trisomy (dup(17)(p11.2p12)) patient was identified and evaluated for the CMT1A phenotype. Electrophysiological measurements indicated decreased motor nerve conduction velocities in this patient, indicating this patient did have CMT1A. Molecular analysis demonstrated that the duplication in the partial trisomy patient completely encompassed the CMT1A duplication and included markers both proximal and distal to the CMT1A duplication (Lupski et al, 1992). This strongly suggests a gene dosage effect is responsible for the electrophysiological phenotype associated with CMT1A. The fact that the partial trisomy 17 duplication extends beyond the CMT1A duplication strongly argues against a model of interruption of a candidate CMT1A gene at the duplication junction or position effect. A model of occurence of a stable dominant

mutation in a duplicated gene contained within the CMT1A duplicated region is also highly unlikely given the *de novo* nature of the cytogenetic abnormality in the partial trisomy 17 patient (Lupski et al, 1992).

The *trembler* and *trembler-J* allelic mouse mutations exhibit a peripheral neuropathy with many similarities to CMT1A. A gene encoding a peripheral myelin protein (*pmp22*) has been mapped to mouse chromosome 11, the same region the *trembler* mutations have been mapped to, a region of conserved synteny with human chromosome 17. Human *PMP22* has been mapped to chromosome 17, within and not disrupted by the CMT1A duplication, suggesting that overexpression of the gene is a likely cause of CMT1A (Patel et al, 1992; Valentijn et al, 1992; Timmerman et al, 1992; Matsunami et al, 1992). The *PMP22* gene is deleted in HNPP patients, suggesting that decreased expression of *PMP22* is also deleterious (Chance et al, 1993).

Methods to study aneuploidy

There are several methods available that can be used to investigate aneuploid syndromes. Since this work focuses on a chromosomal deletion, this discussion will consider deletions only, although it may also be applied to duplications. The first method is to isolate the deleted chromosome in hamster/human somatic cell hybrids. In order to construct somatic cell hybrids containing chromosome 22, the ADE$^-$I Chinese hamster ovary (CHO) selection scheme was developed (van Keuren et al, 1987). This Chinese hamster ovary cell line is deficient in adenylosuccinate lyase (ADSL)

activity. This cell line cannot grow in media devoid of adenine, however the deficiency can be corrected by the human ADSL gene located on chromosome 22. However this selection scheme is no longer used since it leads to extensive breakage and rearrangement. Recent efforts to create somatic cell hybrids containing a rearranged chromosome 22 have utilized the HPRT selection scheme, in which an X chromosome is always retained and the rearranged chromosome 22 is retained by chance. The advantage of capturing a rearranged chromosome in a hybrid is that it allows the use of a simple Southern blot to determine the presence or absence of probes in the deleted region. Disadvantages include the required cytogenetic analysis, the secondary rearrangements that may occur in human chromosomes maintained in hybrids (Epstein et al, 1991), the fact that there is no reliable selection scheme for chromosome 22, the fact that it is extremely laborious, especially for multiple patients and that there are no guarantees that the rearranged chromosome 22 will be isolated from the normal chromosome 22 since this occurs by chance.

The second method that can be used to study chromosomal deletions is *in situ* hybridization. *In situ* hybridization can be used to initially localize a particular probe on a chromosome or, once the probe has been localized, test whether that particular probe is present or absent from the deleted chromosome. The traditional *in situ* hybridization technique used tritium-labeled probes. This allows the probe sequence to be short (as small as 0.4-0.5 kb), but it requires long autoradiographic exposures (usually 5-7 days), examination of 50-100 cells, and extensive statistical analysis. Also, the exact site of hybridization on the chromosome is usually not

evident because of grain scatter and emulsion thickness (Epstein et al, 1991). The newer non-radioactive technique of fluorescent *in situ* hybridization (FISH) offers improved speed, precise localization of the probe to both chromatids, and requires examination of fewer cells. An added advantage is that subtle chromosome rearrangements, such as cryptic translocations can be detected more easily (Kuwano et al, 1991).

In FISH, the probe of interest is biotin labeled and detected using fluorescein isothiocyanate-conjugated avidin (FITC). The signal can be amplified with one additional layer of anti-avidin antibody. The chromosomes are then counterstained with propidium iodide (Pinkel et al, 1986). It is also possible to suppress highly repetitive sequences, such as the Alu or Kpn elements, by adding an appropriate competitor DNA, usually human Cot-1 placental DNA. This exploits the rapid reassociation kinetics of repetitive sequences and allows selective hybridization of unique sequences (Lichter et al, 1990).

There are several factors that influence the intensity and specificity of the hybridization signal, especially the purity of the DNA probe and the size of the probe fragments used in the hybridization reactions (Lichter et al, 1990). FISH generally requires a longer probe sequence (20-40 kb) than the isotopic *in situ* techniques. In addition, the position of the probe on the chromosome and the degree of chromosome condensation affect the resolution of FISH. Elongated, prometaphase chromosomes give higher resolution and show less variability than more compact metaphase chromosomes. Regions of the chromosome that include the

centromere and telomeres often show some variability in position compared to probes in other chromosomal locations, suggesting the possibility that the local chromatin structure plays a significant role in determining the limits of resolution (Lichter et al, 1990). Despite these limitations, FISH is rapidly becoming the method of choice in the diagnosis of small deletions.

The third method used to study chromosomal deletions is Southern blot dosage analysis. This technique allows assessment of copy number of any unique chromosome sequence. Restriction enzyme-digested aneuploid and diploid DNAs are electrophoresed, Southern blotted and, hybridized simultaneously with one or more test probes from the potentially deleted region as well as a reference probe outside that region. The resulting band signals are quantified by densitometry. This process is repeated with a series of probes from the deleted chromosome until the full extent of the deleted and non-deleted regions is defined (Epstein et al, 1991). There are a number of technical considerations that must be taken into account when this type of analysis is performed. The reference probe, located on the same or a different chromosome, should be hybridized simultaneously with the test probe. The test and reference probes should produce bands of a similar size to avoid problems of differential transfer of the large and small fragments during the blotting procedure. It is important to avoid partial enzyme digestions, and multiple replicates of both aneuploid and diploid DNA should be compared. Autoradiograph exposures should be carried out to generate bands for densitometric analysis that are in the linear range of the film. Finally, statistical analysis should be

performed on the results (Epstein et al, 1991). This method is exacting, however it is applicable to the analysis of all DNA sequences and does not require the family members of the proband to be studied.

A variation on the third method, RFLP analysis, may also be useful in determining the dose of a particular probe. Parental DNA is required to show that there is no allelic contribution from one parent. If this is the case, the probe being tested is considered informative and it can be concluded that the probe is deleted in the patient. This method has the added advantage of allowing determination of the parental origin of the deletion, which will allow one to test the possibility of imprinting. However, parental DNA can be difficult to obtain, and without it analysis is limited to the determination of heterozygosity. If a patient is heterozygous for the two alleles, one can conclude that DNA sequence is not deleted, however if only one allele is present it is impossible to tell if it represents the homozygous or hemizygous state without densitometric analysis.

Other deletion syndromes

Several deletion syndromes have been extensively characterized using the methods discussed above. Miller-Dieker syndrome (MDS) is a multiple malformation syndrome characterized by type 1 lissencephaly and a characteristic facial appearance. Lissencephaly is a severe brain malformation whereby the typical covolutions are absent and the brain surface is smooth. Because of occasional familial reoccurence MDS was once thought to follow an

autosomal recessive mode of inheritance. However, it was shown with high-resolution banding that all MDS patients have a chromosomal microdeletion in band 17p13.3. The smallest reported deletion clearly limits the deletion to 17p13.3, and therefore precisely defines the critical region for MDS. Somatic cell hybrids were used to localize several chromosome 17p probes and then RFLP analysis using these highly polymorphic probes was carried out on two cytologically normal MDS patients. It was found that both of these patients had sub-microscopic deletions that allowed a more precise definition of the critical region (vanTuinen et al, 1988).

Recently, a candidate gene (LIS-1, lissencephaly-1) for MDS was found. Two cDNA clones, one identifying the 3' end and the other identifying the 5' end of the gene, were hybridized to a panel of somatic cell hybrids containing a single chromosome 17 from several MDS patients. Results indicated that there was a partial deletion involving only the 5' region in one patient, whereas another patient showed a partial deletion involving only the 3' region of the gene (Reiner et al, 1993). In one of these patients all previous probes failed to detect a deletion. Since these two patients have lissencephaly and the facial dysmorphology of MDS, and their non-overlapping deletions disrupt opposite ends of this cDNA, it seems that mutations of LIS-1 can produce the complete MDS phenotype. The LIS-1 gene encodes a protein related to the β-subunits of G-proteins. G-proteins participate in signal transduction in pyramidal neurons and are also involved in growth cone collapse, a normal process of neuronal migration (Bourne et al, 1993; Igarashi et al, 1993). As deletions in MDS patients render the LIS-1 gene

hemizygous, it appears that half of the normal dosage of the protein product is insufficient for normal brain development (Reiner et al, 1993)

Smith-Magenis syndrome is a clinically recognizable multiple congenital anomaly/mental retardation syndrome due to an interstitial deletion of chromsome 17p11.2. The cardinal features of the syndrome include hypotonia in infancy, short stature, develpomental delay and subsequent mental retardation with speech and language delay greater than motor delay. Self-destructive behavior is common in older individuals. In order to define the deletion interval common to Smith-Magenis patients and to identify patients who had novel deletion intervals, genomic DNA was isolated and analyzed by Southern and RFLP analysis. A total of five markers were deleted in almost all of the Smith-Magenis patients, defining the region critical to the Smith-Magenis phenotype. Of the 31 Smith-Magenis patients studied all but four had only the five markers deleted. The remaining flanking markers were all in 2 copies, indicating that there is not much variation between these patients. It is believed that the patients in this study could be used to construct a deletion mapping panel that could then be correlated to YAC contigs and a PFGE map. This will enable both delineation of specific genes which map to this region and correlation of the genotype with the phenotype in individual Smith-Magenis patients (Greenberg et al, 1991). The markers deleted in Smith-Magenis syndrome are linked to CMT1A, which is interesting because absence of REM sleep is common to both syndromes. This suggests that a gene, involved in sleep function may map to 17p11.2, the region that

borders both Smith-Magenis syndrome and CMT1A (Greenberg et al, 1991).

Wolf-Hirschhorn syndrome is characterized by mental and growth retardation, a distinct pattern of dysmorphic features, and a variety of physical abnormalities. It is caused by a partial deletion of chromosome 4p, and the size of the deletions range from half of the short (p) arm to deletions that are cytogenetically undetectable. The variation in size of the deletion does not result in significant difference in phenotype, suggesting that the relatively few genes responsible for the Wolf-Hirschhorn phenotype are clustered in a defined region of chromosome 4p. The smallest deletion to date was defined in a patient with the typical clinical features of Wolf-Hirschhorn syndrome. The deletion was initially detected by high resolution chromosome analysis and then confirmed by FISH. Using PFGE analysis the size of the deletion was found to be approximately 2.5 Mb in band 4p16.3, refining the location of the critical region (Gandelman et al, 1992).

The fibroblast growth factor receptor-3 (FGFR-3) gene is located within the 2.5 Mb critical region. The FGFR-3 gene has a high level of expression in the brain and plays a role in both neuronal survival and differentiation of the central nervous system (Anderson et al, 1988; Murphy et al, 1990). This gene may play a role in Wolf-Hirschhorn syndrome, since mental retardation is the most consistent feature in this syndrome and approximately one-third of the patients were found to have central nervous system defects on autopsy (Gandelman et al, 1992). The deletion of one copy of FGFR-3 may contribute to some other Wolf-Hirschhorn abnormalities as the

transcript is also expressed in kidney, lung, intestine, testes, liver and other tissues (Thompson et al, 1991). The multiple congenital anomalies in this syndrome may be due to the effect of only one gene or may involve several contiguous genes deleted from one chromosome 4p. It is believed that further molecular characterization of 4p16.3 will facilitate the identification of the gene(s) responsible for Wolf-Hirschhorn syndrome (Gandelman et al, 1992).

Cytogenetic analysis of the WAGR (Wilm's tumor, aniridia, genitourinary abnormalities, mental retardation) syndrome have revealed the frequent presence of a deletion in one chromosome 11 homologue. The deletion always includes chromosome band 11p13. Thus, the 11p13 region has been extensively studied in search of the genes responsible. Molecular analysis of WAGR deletions and those in the Wilms tumor themselves led to the cloning of the Wilms tumor gene (WT1), located at 11p13. Wilm's tumor, an embryonic malignancy of the kidney, is also seen in Denys-Drash syndrome, characterized by intersex disorders, renal insufficiency as well as Wilm's tumor (Breuning et al, 1992). The WT1 gene encodes a protein with four zinc fingers. Genotype analysis of the WT-1 gene in two male children with Wilm's tumor and genital system malformations demonstrated that these individuals have mutations within the WT-1 gene. In addition, ten individuals with Denys-Drash syndrome have been identified with germline nucleotide substitutions within the zinc finger domains of the WT-1 gene (Pelletier et al, 1991). These results provide genetic evidence that germline mutations of WT-1 are responsible both for the Wilm's

tumor predisposition and urogenital malformations seen in WAGR patients.

The positon of the aniridia locus has been defined by a patient with only that defect and several candidate clones have been isolated. One candidate is the homologue of the mouse Pax-6 gene which has been shown to be mutated in the mouse *Small-eye* mutants. A sub-microscopic 11p13 deletion was detected in a child with aniridia and Wilms tumor. Using FISH it was determined that the mother, who also had aniridia, had a deletion that included the aniridia candidate gene that was homologous to Pax-6 and the WT1 gene. FISH analysis was also carried out on patients with isolated aniridia that had a deletion of 11p13. In all of these patients, the Pax-6 homologue was deleted, supporting the idea that the Pax-6 homologue is a strong candidate for the aniridia gene (Fantes et al, 1992). This is one of the first examples in which a deletion syndrome has revealed genes located within that deletion that are directly responsible for the observed phenotype. This is also an example of a contiguous gene syndrome (Schmickel, 1986), in which the complex phenotypic abnormalities associated with a syndrome may result from DNA rearrangements involving several closely linked genes.

Prader-Willi syndrome (PWS) is characterized by reduced fetal activity, hypotonia and feeding problems in infancy, mental retardation, childhood obesity, short stature, hypogonadism and an abnormal facial appearance. A deletion of chromosome 15q11-q13 is found in approximately 60% of PWS patients. A similar 15q11-q13 deletion is found in 60% of patients with Angelman syndrome (AS).

AS is characterized by ataxic movements, seizures, mental retardation, absent speech, frequent laughter and specific facial characteristics. Microscopically, AS deletions appear larger than PWS (Knoll et al, 1989). Although cytogenetically visible interstitial deletions in PWS and AS are similar in size when tested with DNA probes, the critical regions of deletion for the 2 syndromes are being refined through the analysis of patients with unbalanced translocations and sub-microscopic deletions. Current evidence suggests that the critical regions for the two syndromes are distinct (Knoll et al, 1993). More precise definition of the critical regions is needed in order to understand the genes and regulatory elements responsible for the pathogenesis of PWS and AS.

A similar situation may exist between DiGeorge syndrome (DGS) and velo-cardio-facial syndrome (VCFS), both described earlier. The spectrum of anomalies in VCFS when compared to those in DGS, particularly the cardiac anomalies, has led to the suggestion that there is considerable phenotypic overlap between the two syndromes. This has led to the discovery that both syndromes are associated with similar deletions of chromosome 22q11.21-q11.23. Three loci, D22S75, D22S66 and D22S259 have been shown to be deleted in 14 patients with DiGeorge syndrome (Driscoll et al, 1992a). These loci were subsequently shown to be deleted in 14 patients with VCFS (Driscoll et al, 1992b), suggesting that DGS and VCFS share, at least in part, a common pathogenesis. These results have been confirmed by another group with additional DGS and VCFS patients (Carey et al, 1992; Kelly et al, 1993). The common embyonic origin of the affected structures (third and fourth pharyngeal

pouches) in both syndromes suggests that they could result from an abnormal function of the neural crest (Oster et al, 1983). It is possible that DGS is caused by hemizygosity for a different gene, or overlapping genes to VCFS. The genes involved would be closely linked at chromosome 22q11 and could be members of the same gene family. Alternatively, DGS and VFCS could be part of a spectrum of abnormalities which may be caused by monosomy for 22q11 (Kelly et al, 1993). This is supported by the isolated cardiac defects that were also shown to be associated with a deletion of chromosome 22q11, in the same region that is deleted in DGS and VFCS.

In all of the syndromes mentioned above a critical region has been proposed. A critical region is visualized as being the smallest chromosome segment which, when unbalanced, will give rise to the phenotype generally associated with imbalance of a larger chromosomal region that contains it (Epstein, 1986). Presumably, since it is possible to to decompose the phenotype of a chromosomal syndrome into a set of sub-phenotypes that can be mapped to sub-segments of the region of imbalance, critical regions should exist.

In many aneuploid syndromes there seems to be a lack of relationship between the length of the aneuploid segment and the clinical manifestations or severity of the syndrome. Examples where this is the case include Wolf-Hirschhorn syndrome (Gandelman et al, 1992) and Angelman syndrome (Knoll et al, 1993). It is as though, in these cases, the whole phenotype is entirely attributable to the imbalance of only a small chromosome region, and imbalance of the surrounding region is irrelevant. However, it is important to note

that even though certain chromosomal segments do not have significant impacts on the phenotype, this does not mean that they may not have more subtle effects (Epstein, 1986). An example is Down syndrome in which the distal part of 21q22.1 and proximal part of 21q22.3 seems to be all that is required to cause the physical phenotype of Down syndrome. However, duplication of chromosome 21pter-q22.1 is often associated with some degree of mental impairment, therefore imbalance of both the proximal and distal parts of 21q probably contributes to the overall degree of mental retardation in Down syndrome (Epstein, 1990). Thus, while the concept of a critical region or regions may be useful for identifying the part or parts of a chromosome that seem to have the greatest effect on the observable phenotype, it is important not to fall into the trap of concluding that it is the imbalance of only this region or regions that has deleterious effects on development or function (Epstein, 1990).

From these examples it is evident that the utilization of the methods described is feasible for the characterization of chromosomal deletions. In addition, all of the reports support Epstein's notion of phenotypic mapping and the existence of critical regions.

Research summary

Seven patients, all demonstrating similar visible deletions of chromosome 22q13.3, were used to characterize a new deletion syndrome. The phenotypes of these patients were compared and a molecular analysis was undertaken. The extent of the deletions and

the region of the proximal deletion breakpoint in these patients was determined by dosage analysis using a combination of RFLP and densitometric analysis of probes that were previously genetically mapped to the region (Dumanski et al, 1991). An attempt was made to visualize the proximal deletion breakpoint with pulsed field gel electrophoresis in several patients. In addition, several yeast artificial chromosomes were obtained and tested to see if they contained the sequences that represented the deletion breakpoint in two patients. Finally, fluorescent *in situ* hybridization was used to confirm the status of the most distal probe known on chromosome 22 in several patients.

MATERIALS AND METHODS

Clinical evaluation

Seven del(22)(q13.3) patients were identified by high resolution cytogenetic analysis that was performed to determine the cause of developmental delay. Cytogenetic analyses were performed by Ikuko Teshima at the Hospital for Sick Children, Toronto (QM), David Ledbetter at the Baylor College of Medicine, Houston (AJ, JT), Jackie Biegel at the Philadelphia Children's Hospital (CH), A. Amir Al Saadl at the Medical College of Ohio, Toledo (NS), and the Genetics Associates of Miami (CB). A clinical description of each patient was compiled by the referring physician and a clinical summary based on the descriptions and patient photographs was compiled by Dr. Jerome Gorski at the University of Michigan, Ann Arbor (clinician for CH).

Cell lines

Cell lines from the seven del(22)(q13.3) patients were obtained after high resolution cytogenetic analysis revealed the deletion. A blood sample was obtained to establish Epstein-Barr virus (EBV) transformed lymphoblastoid cell lines for QM, CH, CB, NS, JT and AJ. A fibroblast culture was established for FB. EBV-transformed lymphoblastoid lines were also established from the parents of AJ and the mother of JT. Fibroblasts were established from the parents of FB. Cell lines were kindly provided by Mary Phelan at the Greenwood Genetic Center, South Carolina (FB), Jacqueline Siegel-Bartelt at the Hospital for Sick Children, Toronto (QM), Jackie Biegel

at the Philadelphia Children's Hospital (CH), Robert Desnick and Anne Wang at Mount Sinai School of Medicine, New York (NS, CB), and Stuart Shapira at the Baylor College of Medicine, Houston (AJ, JT and parents).

Lymphoblast lines were cultured in RPMI 1640 media (Gibco-BRL) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% glutamine. Fibroblast lines were grown in D-MEM media (Gibco-BRL) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% glutamine. The fibroblasts were washed in Hank's balanced salt solution (HBSS) and then trypsinized before transferring to fresh cell culture plates (150 mm^2). All cells were grown at 37°C in 5% CO_2. Aliquots of the cultures were frozen in 5% DMSO/FBS and stored in liquid nitrogen.

DNA Preparation

Liquid:

Cells from approximately 20 T25 flasks (lymphoblastoid cells) or 30 150 mm^2 plates (fibroblasts) were harvested to obtain liquid genomic DNA. Approximately 0.5-2 mg of DNA was obtained on average. Cells were washed three times in 25 ml Hank's balanced salt solution (HBSS) to remove the media by spinning in a clinical centrifuge for 10 minutes at 800 rpm. Cells were resuspended in 4.5 ml 10/10/10 solution (10mM Tris 7.5, 10mM 5M NaCl, 10mM 0.2M EDTA), 0.5 ml 10% SDS and 100 ul 10mg/ml Proteinase K. The lysate was left overnight in a 37°C water bath. DNA was extracted by a phenol/chloroform method (Sambrook et al, 1990). An equal volume

of phenol, phenol/chloroform and chloroform was added in succession. The solutions were mixed carefully to avoid shearing and spun 10 minutes each in a clinical centrifuge. The DNA was precipitated by adding 1/10 volume sodium acetate (3M) and 2-2.5 times volume cold 95% ethanol. The clump of DNA was removed with a plastic pipette tip and was rinsed several times with 70% ethanol. The DNA was spun down in a microfuge for 2 minutes and was then dried to remove the ethanol and resuspended in TE buffer (volume varied with the amount of DNA).

Agarose Plugs:

DNA prepared from cells was embedded in agarose plugs for pulsed field gel electrophoresis based on the yeast protocol of Schwartz and Cantor (1984). Fibroblasts were trypsinized from 10-30 large plates and lymphoblastoid cells were harvested from 5-10 T25 flasks. Cells were washed in HBSS and spun down in a clinical centrifuge (International Equipment Company) (1000 rpm, 10 minutes). The cells were resuspended in 5 ml HBSS and counted with a hemocytometer. Cells were spun again and resuspended in enough HBSS to give a final concentration of 1.4-2.0 x 10^7 cells/ml. An equal volume of 1% InCert agarose (FMC) was added to the cells, mixed and pipetted into a plug-former (BioRad). The plugs were allowed to solidify on ice for 10 minutes and then were placed in 25 ml ESP (0.5M EDTA, 1% sodium laurosyl sarcosine, 1 mg/ml Proteinase K) to lyse the cells and digest the proteins. The plugs were incubated at 50°C for 2 days, then stored at 4°C. Before the DNA could be digested with restriction enzymes, the plugs were

washed for one hour each in the following solutions: two times in TE + 1mM PMSF and three times in TE alone. The plugs were then stored at 4°C in TE until required.

Probes

The probes used in this study, their chromosomal location, the restriction enzyme displaying polymorphism and expected allele sizes are summarized in Table 2. The probes ARSA (Herzog et al, 1990) and PDGFB (Ratner et al, 1985) identify the arylsulfatase A gene and the platelet derived growth factor gene respectively. The remaining probes recognize anonymous DNA sequences.

The probes D22S102, D22S90, D22S91, D22S92, D22S95, D22S97, D22S94, D22S22, D22S82, D22S84, and D22S21 are all contained in Charon 21A phage and were isolated from a chromosome 22-specific genomic library constructed from a flow-sorted chromosome 22 (Dumanski et al, 1990; Rouleau et al, 1989). Large scale phage preparations were used to isolate the DNA (modified from Sambrook et al, 1990). An overnight culture of LE392 cells was grown at 37°C. 150 ul of primary phage lysate was added to 1 ml of LE392 cells. This was then added to 250 ml NZ broth and grown overnight at 37°C. Ten grams of NaCl was added to the lysed cells which were then centrifuged at 5000 rpm for 10 minutes at 4°C. The supernatant was kept and 25 g PEG8000 was added to it. This was left at 4°C overnight. The phage were then centrifuged at 5000 rpm for 10 minutes at 4°C. The pellet was resuspended in 4 ml λdil (100 mM NaCl, 10 mM Tris pH 7.5, 10 mM MgCl2) and a chloroform extraction was performed to remove much

Table 2
Description of the Polymorphic, Chromosome 22-Specific Markers Used in the Present Study

Locus (probe)	Location*	RFLP enzyme	Constant bands	Allele sizes	Reference
D22S102 (KI-436)	22q12-q13	TaqI	5	4.1/3.8	Dumanski et al. 1990
D22S90 (KI-185)	22q12-q13	TaqI	-	7.7/6.3+1.4	Dumanski et al. 1990
D22S91 (KI-211)	22q12-q13	MspI	2.5, 1.4, 1.3 0.9, 0.7	1.95/1.1	Dumanski et al. 1990
PDGFB (pSM-1)	22q12.3-q13.1	HindIII	-	21/14.5+6.5	Ratner et al. 1985
D22S92 (KI-218)	22q12-q13	MspI	0.75	0.7/0.6	Dumanski et al. 1990
D22S95 (KI-839)	22q13-qter	EcoRV	-	20/15	Dumanski et al. 1990
D22S64 (pH130)	22q13-qter	PvuII	1.8	1.6/1.5	Budarf et al. 1991
D22S40 (pH19)	22q13-qter	HindIII	-	11.7/6.2	Budarf et al. 1991
D22S97 (KI-260)	22q13-qter	TaqI	2.3	2.6/2.4	Dumanski et al. 1990
D22S94 (KI-1105)	22q13-qter	TaqI	-	6.6/5.5	Dumanski et al. 1990
D22S22 (W110D)	22q13-qter	TaqI	2.2	1.9/1.8	Rouleau et al. 1989
D22S82 (KI-63)	22q13-qter	TaqI	1.2	3.2/1.6	Dumanski et al. 1990
D22S84 (KI-216)	22q13-qter	PvuII	-	3.9/2.2+1.7	Dumanski et al. 1990
D22S45 (pH41a)	22q13-qter	PvuII	1.8	A1 2.7 A2 2.6 A3 2.1	Budarf et al. 1991
D22S21 (W13E)	22q13-qter	TaqI	1.05	A1 2.8 A2 1.6 B1 1.5 B2 1.35	Rouleau et al. 1989
D22S55 (pH91)	22q13.3-qter	TaqI	2.2, 0.56	A1 1.55 A2 1.40 A3 1.35 A4 1.30 A5 1.25	Budarf et al. 1991
ARSA (pCP8)	22q13.3-qter	TaqI	1.1	3.9/3.2	Herzog et al. 1990

*- Determined by somatic cell hybrid mapping except for D22S22 and D22S21 which were mapped by genetic linkage

of the protein. A cesium chloride gradient was established by layering 0.5 ml 1.7 CsCl, 1.0 ml 1.5 CsCl and 1.0 ml 1.3 CsCl. The 4 ml phage pellet was carefully layered on top of the CsCl gradient. This was spun at 35000 rpm at 20°C for 60 minutes in a Beckman ultracentrifuge (Ti50 fixed angle rotor). The phage band was removed and mixed with an equal volume of saturated CsCl. A further 3.0 ml 1.5 CsCl and 1.0 ml 1.3 CsCl was layered on top and the gradient was spun at 35000 rpm at 20°C for 60 minutes. The phage band was removed, placed in Spectra-Por dialysis tubing and dialysed at 4°C overnight against 20mM Tris pH8.0, 20mM NaCl, 1mM $MgCl_2$. The phage were removed from the dialysis tube and 5 ul/ml 20% SDS and 50ul/ml 200mM EDTA was added to it. The mixture was incubated at 65°C for 10 minutes. Proteinase K (50ug/ml) was added and the mixture was allowed to incubate 30-60 minutes at 37°C. The DNA was extracted with an equal volume of phenol, phenol/chloroform, and chloroform. The DNA was precipitated by the addition of 20ul/ml 5M NaCl and an equal volume of isopropanol. The DNA was washed in 500 ul 70% ethanol and the pellet was dried and resuspended in 200-1000 ul TNE. The concentration was estimated by visualization on a gel, and approximately 100-500 ug of DNA was obtained per 250 ml culture.

Plasmid and Cosmid Preparations

Several of the probes used were contained in plasmids or cosmids. The probe identifying locus PDGFB was isolated from the plasmid pSM-1 (Ratner et al, 1985), the probes identifying loci D22S64, D22S40, D22S45, D22S55 were subcloned in pUC18 (Budarf

et al, 1991) and the probe identifying ARSA was subcloned in Bluescript SKM13- (Stein et al, 1989). The cosmids used were isolated from the chromosome 22-specific arrayed cosmid library LL22NCO3 in vector Lawrist16. The cosmids N32H10 and N72F5 both recognize the locus D22S64 (address kindly provided by Ian Dunham, London, England), and N73A6 recognizes ARSA (address kindly provided by James Troffater, Harvard).

Large scale DNA preparations were accomplished using the Promega Magic Maxi Preps. Briefly, 250 ml of the plasmid or cosmid was grown in LB plus the appropriate antibiotic (ampicillan for plasmids, kanamycin for Lawrist cosmids). The cells were pelleted by centrifugation (8000 rpm, 15 minutes) and resuspended in 15 ml cell resuspension solution (50 mM Tris pH 7.5, 10 mM EDTA, 100 ug/ml RNase A). Cell lysis solution (0.2M NaOH, 1% SDS) was added and mixed gently until the solution became viscous. An additional 15 ml neutralization solution (2.55M potassium acetate, pH 4.8) was added, the cells were mixed by inversion and centrifuged (8000 rpm, 15 minutes). The cleared supernatant was filtered through a sterile J-cloth and 0.6 volumes isopropanol was added. This was centrifuged at 8000 rpm for 15 minutes. The DNA pellet was resuspended in 2 ml TE buffer. The DNA purification solution (7M guanidine HCl) was added to the DNA and this was passed through the provided columns by applying a vacuum. Column wash (200mM NaCl, 20 mM Tris pH 7.5, 5mM EDTA, diluted 1:1 with 95% ethanol) was added to the column several times and the column was then rinsed with 5 ml 80% ethanol. The column was allowed to dry and a final 1.5 ml TE buffer (pre-heated to 65oC) was added. The column

was spun in a clinical centrifuge for 5 minutes at 2000 rpm and the eluate collected. The DNA was stored at -20°C.

Dosage analysis

Restriction enzyme digests and gel electrophoresis:

The DNA was digested with restriction enzymes for RFLP analysis (various) or densitometric analysis (HindIII or EcoRI) according to the manufacturers directions (Gibco-BRL, Pharmacia, NEB). The genomic DNA digests were incubated for 7-16 hours. RNase was added to a concentration of 0.05 ug/ml after the restriction enzyme digest to remove contaminating RNA, as was 1/10 volume OrangeG in 20% Ficoll and 100mM EDTA. This was incubated at 65°C for 10 minutes to allow the RNase to act and to stop the enzyme digestion. DNA was separated by electrophoresis on 0.8% agarose gels, made and run in TBE buffer (0.089M Tris, 0.089M Boric acid, 0.002M EDTA, pH 8.4). The gel was then stained in ethidium bromide (final concentration of approximately 0.5 ug/ml) to visualize the DNA. The ethidium bromide was made to fluoresce by placing the gel on a transilluminator UV box (Fotodyne Incorporated).

Transfer of DNA to membrane:

Two methods were used to transfer the DNA to GeneScreen Plus (DuPont) membrane. The first was by the standard capillary method (Southern, 1975). The gel was denatured in 0.5 M NaOH, 1.5 M NaCl twice for 30 minutes each at room temperature with gentle agitation. The gel was rinsed briefly in ddH20 and transferred to a neutralizing solution (1.5 M Tris, 1.5 M NaCl, pH 7.5) for two 30

minute intervals with gentle agitation. A piece of membrane the exact size of the gel was cut out and soaked in 10x SSC (1.5 M NaCl, 0.15 M Nacitrate) for 15 minutes. The transfer was set up by placing 3 pieces of Whatmann 3MM filter paper on a glass plate such that the ends of the filter paper hung into a reservoir of 10x SSC. The gel was placed face down on the filter paper and the membrane was placed on top of the gel. A further 3 pieces of 3MM filter paper were placed on top of the membrane. A stack of blotting paper (Schleicher and Schull) was placed on top and the entire set-up was covered in plastic wrap to prevent evaporation of the SSC. The transfer was allowed to proceed overnight. The membrane was then washed in 0.4 M NaOH for 60 seconds and neutralized in 0.2 M Tris, 2x SSC for 5 minutes. The membrane was allowed to dry completely before hybridization.

The second method of transfer was by use of a vacuum blotter (BioRad). A piece of membrane, cut to the size of the gel was placed on a piece of 3MM filter paper which was placed in the center of the vacuum blot apparatus. A plastic mask was placed on top of the membrane, such that all but 1 cm around the edge of the membrane was exposed. The gel was placed on top of the mask and a vacuum was applied (approximately 2.5 mm of Hg). The following solutions were then poured on top of the gel: denaturing solution (0.5M NaOH, 1.5M NaCl) for 20 minutes, neutralizing solution (1.5M Tris, 1.5M NaCl) for 15 minutes, and 10x SSC for 60 minutes. The membrane was then placed in the wash solutions as described above. For efficient transfer of large pieces of DNA (>20 kb), a depurination step was included before denaturation. The gel was placed in 0.25 M HCl

for 10 minutes to "acid-nick" the DNA. The gel was thoroughly rinsed with distilled water before proceeding to the denaturation step.

Hybridization:

All DNA probes were digested with appropriate restriction enzymes, purified in low-melting point agarose (SeaPlaque-FMC) by gel electrophoresis, and used without further purification. DNA probes were labelled with α-32PdCTP using the random primer method (Feinberg and Vogelstein, 1983). Briefly, an oligo labelling reaction was set up by adding 10 ul OLB (100 ul 1 M Tris, 12.5 ul 1 M MgCl2, 2.5 ul 50 mM dATP, 2.5 ul 50 mM dGTP, 2.5 ul 50 mM dTTP, 250 ul 2 M Hepes pH 6.6, 150 ul 90 A260 units/ml random primers, 2 ul β-mercaptoethanol) and 2 ul BSA (10 mg/ml) to approximately 25-50 ng DNA that has been denatured by boiling for 10 minutes. To this 1.5 ul (15 units) Klenow fragment (Pharmacia) and 5-10 ul (50-100 uCi) α-32PdCTP (ICN) was added to give a final volume of 50 ul. The reaction was incubated at 37°C for a minimum of two hours. The reaction was terminated by adding approximately 15 ul "stop buffer" (blue dextran in 0.2M EDTA). The mixture was passed over a G-50 Sephadex column equilibrated in TE to remove unincorporated label. The eluate was boiled for 10 minutes before adding to the membrane. Certain labelled probes were pre-annealed for four hours at 65°C with sonicated placental DNA to repress repetitive elements (Litt and White, 1985). Hybridization was performed in 7% SDS, 0.263M sodium phosphate, 1mM EDTA, 5x Denhardt's solution and denatured herring sperm DNA (a slight

modification of Church and Gilbert, 1984) at 65°C in a hybridization oven (Tyler). Filters were washed twice for 5 minutes each at low stringency (2x SSC, 0.1% SDS at room temperature), twice for 10 minutes each in 0.2x SSC, 0.2% SDS at 65oC, and once for 10 minutes in 0.1x SSC, 0.2% SDS at 65°C. Filters were exposed to Kodak XAR-5 film at -70°C for varying lengths of time.

RFLP and Densitometric analysis:

RFLP analysis was carried out on every patient for most probes to determine the copy number. In most cases the DNA from the parents was not available, therefore analysis was limited to whether or not the patient was heterozygous for a particular probe. If RFLP analysis was uninformative, quantitative analysis of Southern blots was performed to determine copy number. A data set consisted of two del(22)(q13.3) patients and a normal control. The data set was repeated four times on a single gel. The resulting blots were simultaneously hybridized to both the test probe and one of two control probes which should be in 2 copies in all individuals tested; H20 localized to 22q11-q12 (Budarf et al, 1991), or GSH8, localized to 21q22.2-q22.3 (Stewart et al, 1985).

Statistics:

The hybridization signal was quantified using a GelScan LKB 2400 Laser Densitometer or the BioRad Model GS-670 Imaging Densitometer. The results were analyzed statistically using the Wilcoxon Rank-Sum test (Wilcoxon and Wilcoxon, 1964) to compare dosage between the two patient and the control DNA. The Wilcoxon

Rank-Sum test is a nonparametric test in which the null hypothesis is not concerned with specific parameters, but only with the distribution of the variants. In order to apply the Rank-Sum test, the density (measured by area) of the bands for the test probe and the control probe were obtained for the normal control and the patients. The density of the band for the test probe was divided by the density of the band for the control probe, producing a ratio for each lane. The ratios from the control DNA and one patient DNA were compared. The lowest ratio was given a rank of 1 and the remaining ratios were ranked in order from smallest to largest. The ranks for the control DNA and the test DNA were totaled and compared using a table of critical values for the Wilcoxon Rank-Sum test (Verdooren, 1963). A deletion was indicated if the rank sum for the patient DNA was significantly lower than that of the control. A p-value from the table of 0.025 (97% confidence) or less was considered a significant difference between the control and the patient. To confirm that the patient values were half that of the control, the ratios from the patient were doubled and compared to the control by again assigning ranks to the doubled patient ratios and the original control values. The ranks were totaled and compared on the table of critical values. If the difference between the control and the patient was now insignificant (p-value>0.10, 90% confidence) it was concluded that the patient DNA had one copy of the test probe and was therefore deleted at this locus.

Pulsed field gel electrophoresis

Agarose plugs containing genomic DNA were digested with the appropriate rare-cutting restriction enzyme in the following manner. An appropriately sized plug was placed in a 1.5 ml microfuge tube with 20 ul BSA, 20 ul restriction enzyme buffer and enough sterile water to make the final volume 200 ul. The tubes were left on ice 2 hours to allow the buffer to saturate the plugs. The appropriate restriction enzyme was then added (10 units) and the tubes were placed at 4°C for three hours to allow diffusion into the plugs. A further 10 units restriction enzyme was added and the plugs were incubated at the appropriate temperature (usually 37°C) overnight. The plugs were then washed in 0.5x TBE 4 times for 60 minutes each, with the buffer changed every hour. The plugs were loaded into a 1% agarose gel made with 0.5x TBE and sealed into the wells with 1% low melt agarose in 0.5x TBE. A wild-type lambda concatamer ladder standard and a yeast chromosome standard were also loaded into the gel (New England Biolabs). The DNA was separated using a BioRad CHEF DR-II system. The gel was run in 0.5x TBE under the following conditions: 60 second pulse for 15 hours followed by a 90 second pulse for 9 hours at 200 volts. The gel was stained in ethidium bromide for 30 minutes and destained for several hours before transferring to a nylon membrane as previously described.

Yeast Artificial Chromosomes

YACs containing sequences recognizing D22S95 were obtained courtesy of Ian Dunham, Guy's Hospital, London, England. YAC A21F12 comes from the Washington University library (Brownstein et al, 1989). The YACs 109E6 and 137C6 come from the ICRF library

of Hans Lehrach (Larin et al, 1991). The YAC clones were grown in 5 ml YCD media lacking tryptophan and uracil for 72 hours at 30°C. The cells were spun in a clinical centrifuge (1000 rpm, 10 minutes) and the pellet was resuspended in 500 ul spheroplasting solution (SCE=1M Sorbitol, 0.1M citrate pH 5.8, 10 Mm EDTA). This was incubated at 37°C for 60 minutes. An equal volume of 1.2% low melt agarose (SeaPlaque- FMC) in SCE was added to each tube. The mixture was dispensed into a plug-former and placed on ice for 10 minutes to allow the plugs to solidify. The plugs were then placed in ESP (9.0 ml 0.5M EDTA, 1.0 ml 10% sarcosyl, 100 ul 1M Tris pH 7.5, 10 mg Proteinase K) and incubated at 50°C for 48 hours. The ESP solution was replaced with 0.5M EDTA and the plugs were stored at 4°C. The yeast chromosomes were separated by pulsed field gel electrophoresis as described above.

Cytogenetics

Preparation of metaphase spreads:

Fresh cell cultures were grown for each patient as described above. The following methods were adapted from Watt and Stephen (1986). Cells were fed 24 hours prior to the addition of colcemid to stimulate division of cells. Colcemid was added to each flask at a final concentration of 0.2 ug/ml. The flasks were incubated for 60 minutes at 37°C, then harvested as outlined below. Alternatively, 0.45 ug/ml methotrexate, a cell cycle inhibitor that causes a G1/S interface block, was added to each flask of cells that had been fed within the previous 24 hours, and incubated overnight (16 hours). To remove the block, a final concentration of 0.24 ug/ml thymidine

was added and the cells were incubated for a further 5 hours, 10 minutes. Colecimid (0.05 ug/ml) was added for the last 10 minutes. To harvest, the cells were then centrifuged (1000 rpm, 10 minutes) followed by resuspension in 10 ml 0.075M KCl. The cells were incubated 10 minutes at 37°C and then centrifuged (1000 rpm, 10 minutes). The cells were fixed in 10 ml 3:1 methanol:glacial acetic acid, with the first 2 ml added very slowly. Cells were again spun 10 minutes at 1000 rpm and fixed twice more. The cell suspension was dropped from a height of approximately 1.5 meters onto clean slides that had been pre-chilled in 4°C ddH2O. Slides were placed in the humid air of a 37°C water bath to help spread the chromosomes.

Fluorescence *in situ* hybridization

Biotin labelling:

The fluorescent *in situ* hybridization was carried out as outlined by the Oncor kits. Before any cosmid probes were biotin labelled, they were first cut with Sau3A to make the nick translation more efficient. A digest containing approximately 1 ug DNA, 2ul BSA, 2ul Sau3A buffer, 2 ul (8 units) Sau3A (NEB), and ddH2O to 20 ul was set up and allowed to incubate at 37°C for 90 minutes. The DNA was precipitated by the addition of 1/10 volume sodium acetate and 2x volume 95% ethanol. This was placed at -70°C for 30 minutes and spun in a microfuge for 3 minutes. The supernatant was discarded and the DNA pellet was resuspended in 35 ul TE buffer. The following labelling reaction was then set up: 35 ul Sau3A cut DNA probe, 10 ul nucleotide mixture containing the biotin labelled nucleotide (Oncor), and 5 ul nick translation enzyme mixture (Oncor).

This was mixed well and placed at 15°C for 75 minutes. To stop the reaction 50ul 50mM EDTA was added. The biotin labelled probe was stored at -20°C.

Pre-annealing repetitive sequences:

50-100 ng labelled probe was added to 50-100 times excess human Cot1 DNA which was used as a competitor DNA to suppress the repetitive sequences (Lichter et al, 1990). The labelled probe/Cot1 DNA was precipitated by the addition of 1/10 volume 3M sodium acetate (pH 4.5) and 2-2.5 times 95% ethanol followed by placing it at -70°C for 30 minutes. The precipitated DNA was spun down in a microcentrifuge and the supernatant was removed. To the pellet 6 ul formamide, 1.2 ul 20x SSC, 2.4 ul 50% dextran sulfate and 2.4 ul ddH_2O was added. The probe was placed at 37°C for 2 hours to dissolve and was pipetted every 30 minutes to ensure that the probe was well resuspended. The probe was placed in a 75°C water bath for 5 minutes for denaturation and then was transferred to a 37°C water bath for 1.5 hours.

RNase treatment of slides:

Cells were dropped onto clean slides as described above. The slides were treated with 70 ul RNase (100 ug/ml in 2x SSC). A glass coverslip was added and the slides were placed in a humidifying chamber at 37°C for 30-60 minutes. A humidifying chamber consists of a square plastic petri plate that contains a piece of moist filter paper. Several glass microscope slides were taped together to make two stands that were placed on top of the filter paper. The

slides were washed 3 times with 2x SSC in Coplin jars (2 minutes each) and dehydrated through cold 70%, 95%, and 100% ethanol washes (2 minutes each).

Denaturation of slides:

Denaturing solution (70% formamide, 2x SSC) in a Coplin jar was preheated to 70°C in a water bath. Slides (no more than two at a time) were denatured for two minutes. The slides were then dehydrated through 70%, 95%, and 100% ethanol washes (2 minutes each). Slides were allowed to air dry then were pre-warmed to 37°C. The probe was applied to the slide, a clean glass coverslip was added and sealed with rubber cement. The slide was placed in a humidifying chamber at 37°C for 16 hours.

Washing of slides:

The slide was put through the following washes:

-50% formamide, 1x SSC, 2x 15 minutes, 45°C, shaking;

-1x SSC, 2x 10 minutes, 45°C, shaking;

-0.1x SSC 1x 5 minutes, 45°C, shaking;

-0.1x SSC, 1x 5 minutes, room temperature.

The slide was then transferred to BT-NaCl (0.1 M sodium bicarbonate, 0.3M NaCl, 0.05% Triton-X) for 2 minutes.

Detection:

To the slide, 70 ul blocking agent (3% BSA, 4x SSC) was added. A plastic coverslip was placed on the slide and the slide was allowed to incubate for 10 minutes at room temperature. The coverslip was

removed and 70 ul avidin-FITC (1:400 dilution) was added with a plastic coverslip. The slide was placed in a humidifying chamber at 37°C for 20 minutes. The slide was washed in pre-warmed 45°C BT-NaCl in Coplin jars 1x 10 minutes, 2x 5 minutes.

Amplification:

Normal goat serum (1:10 dilution in 4x SSC) was added to the slide with a plastic coverslip and incubated 10 minutes at room temperature. The coverslip was removed and 70 ul anti-avidin-FITC (1:400 dilution in 4x SSC) was added. A plastic coverslip was also added and the slide was incubated at 37°C for 20 minutes. The slide was washed at 45°C in BT-NaCl 1x 10 minutes, 2x 5 minutes. The slide was treated with 70 ul blocking agent for 5 minutes at room temperature. A further 70 ul avidin-FITC (1:400 dilution in 4x SSC) was added. The slide was incubated at 37°C for 20 minutes with a plastic coverslip. The slide was washed at 45°C in BT-NaCl 1x 10 minutes, 2x 5 minutes.

Counterstaining:

Propidium-iodide with "antifade" (18 ul) was added to the slide. A clean glass coverslip was also added and the antifade was allowed to spread for 2-3 minutes. The slide was wrapped in a Kimwipe to remove any excess solution. The slide was viewed using a Zeiss Axiophot photomicroscope. A Zeiss filter 9 was used which produces blue excitation from 450-490 nm. The chromatic beam splitter allowed wavelengths of less than 510 nm to pass through the objective and the barrier filter only allowed wavelengths of greater

than 520 nm to pass through to the eyepiece. Photographs were taken with Kodak Ektachrome 100HC colour slide film.

RESULTS

Clinical Analysis

The present study consists of seven del(22)(q13.3) individuals, four males and three females. At their last medical exam, their ages ranged from 2 to 5 years. Photographs of six of the cases are shown in Figure 1. A photograph of the seventh patient, FB, has been published (Phelan et al, 1992).

The patients shared a number of clinical symptoms as outlined in Table 3. All seven showed hypotonia and developmental delay. The developmental delay showed a number of similarities. At 26 months FB was at the 10-12 month level of development and was able to take several steps unassisted at 35 months. Similarly, at 60 months QM was at the 30-36 month level for gross and fine motor skills and was not able to take unassisted steps until 35 months. CB walked unsupported at 39 months and at 29 months AJ was just beginning to take single steps. NS walked with support at 26 months while JT walked unassisted at 24 months. In addition, all patients demonstrated relatively severe delays in expressive speech. At 45 months FB was babbling but said no words. At 60 months QM was unable to speak but could sign. At 42 and 29 months respectively, CB and AJ were not vocal but AJ babbled. At 26 months NS babbled but said no words and JT had a vocabulary of approximately 10 words at 56 months.

Two of seven of the children had macrocephaly, which is an unusual physical feature for a deletion syndrome and may have been a consequence of dilatation of the cerebral ventricles. The parents of

Figure 1: Photographs of six del(22)(q13.3) patients at the following ages: QM- 5 years. CH- 4 years, CB- 3 and 6/12 years, AJ- 2 and 5/12 years, NS- 2 and 2 /12 years, and JT- 4 and 8/12 years. The photogaphs were provided by Dr. Jacqueline Siegel-Bartelt at the Hospital for Sick Children, Toronto (QM), Dr.Jerome Gorski at the University of Michigan, Ann Arbor (CH), Dr. Robert Cullen Jr. at the Miami Children's Hospital (CB), Dr. Ted Kurczynski at the Medical College of Ohio, Toledo (NS) and Dr. Stuart Shapira at the Baylor College of Medicine, Houston (AJ, JT).

QM
CH
CB
AJ
NS
JT

Table 3
Clinical Description of Del(22)(q13.3) Patients

Perinatal		
Growth measurements appropriate for gestational age	7/7	
Term gestation	7/7	
Breech delivery	2/7	(CH, QM)
Cesarian section	4/7	(CH, QM, AJ, CB)
Neurological Abnormalities		
Hypotonia	7/7	
Developmental delay	7/7	
Delay of gross motor milestones	7/7	
Delays or absence of expressive speech	7/7	
Macrocephaly	2/7	(CH, AJ?)
Mild dilatation of the cerebral ventricles	4/7	(CH, AJ, NS, CB)
Small anterior fontanel	2/7	(AJ, NS)
Accentuated deep tendon reflexes	1/7	(AJ)
Depressed deep tendon reflexes	1/7	(JT)
Seizures	1/7	(CH)
Dysmorphic Features		
Dolicocephaly	6/7	(CH, FB, QM, CB, JT, AJ)
Ptosis	3/7	(FB, QM, JT)
Epicanthal folds	4/7	(CH, FB, QM, CB)
Dysplastic ears (posteriorly rotated, preauricular pits)	6/7	(CH, FB, QM, AJ, NS, CB)
Downslanting palpebral fissures	1/7	(CH)
Highly arched palate	1/7	(CB)
Simian crease	1/7	(FB)
Syndactyly	1/7	(FB)
Tall stature (>95%)	1/7	(CB)
Large hands and feet (>95%)	1/7	(CH)

Isolated finding limited to a single patient include:

Hyperplastic primary vitreous (QM)
Lymphedema (CH)
Multiple pneumonias (CH)
Esotropia (AJ)
Ventricular septal defect (NS)
Clinodactyly (QM)
Terminal hypoplasia of terminal phalanges (QM)
Gastroesophogeal reflux (FB)

CH had head circumferences well within the normal range, eliminating benign familial macrocephaly in this patient. While the father of AJ showed borderline macrocephaly (58.4 cm, slightly over 98% at 18 years), AJ's macrocephaly was much more pronounced (54.6 cm, >>98% at 45 months). Patient CB also demonstrated macrocephaly with a head circumference of 54 cm (>95% at 54 months) however her father was also macrocephalic (59.5 cm, >98% at 18 years) consistent with a familial origin.

The patients also shared a limited number of dysmorphic features including dolicocephaly, which may have been related to macrocephaly, ptosis, epicanthal folds and dysplastic ears. Most of the patients were of normal stature; none were growth retarded. However two of the patients were disproportionately large for their age; CB was above the 95 percentile in height and CH had disproportionately large hands and feet. The clinical descriptions were done by individual physicians and compiled by Dr. Jerome Gorski (the clinical geneticist of CH) at the University of Michigan, Ann Arbor.

Cytogenetic Analysis

The seven del(22)(q13.3) cases were identified by high resolution cytogenetic analysis in various laboratories after they presented with developmental delay or hypotonia in early childhood. Examination of metaphase spreads with GTG banding revealed a terminal deletion of the long arm of one chromosome 22 in each patient. High resolution analysis revealed that the breakpoint was in band 13.3. The karyotype was designated 46,XY or

XX,del(22)(q13.31) in the case of FB, QM, AJ and JT; and 46,XY or XX,del(22)(q13.3) in the case of CH, CB and NS. High resolution analysis on the parents of all of the patients revealed normal karyotypes, indicating that the deletions were all *de novo*. A partial karyotype of each patient showing one normal and one deleted chromosome 22 is shown in Figure 2.

RFLP Analysis

An RFLP analysis was performed on all of the patients with most of the available probes. Cell lines derived from the deletion patients were analyzed for the presence of heterozygosity, indicating that the particular locus was not deleted. A single allele could represent the homozygous or hemizygous state and was therefore considered uninformative. Figure 3 shows three examples of RFLP analysis. DNA from the seven deletion patients, their parents (where possible), and normal controls was digested with Taq1, EcoRV, or HindIII (Figure 3A, B, and C, respectively). The D22S90 probe produces polymorphic alleles of 7.7 kb and 6.3 kb. The probe was informative in six of the seven cases. FB, QM, CH, NS, AJ, and JT were all heterozygous, indicating that this locus was not deleted in these cases (Figure 3A). The D22S95 probe produces polymorphic alleles of 20 kb and 15 kb. This probe was informative in the case of FB, CH, CB, and AJ, indicating that this locus was not deleted in these patients (Figure 3B). The D22S40 probe, which produces alleles of 11.7 kb and 6.2 kb, was only informative in the case of JT (Figure 3C). The complete results are summarized in Table 4. The D22S102 probe was informative in three cases: FB, QM, and CB were all found to be

Figure 2: Partial karyotypes of six del(22)(q13.3) patients. The partial karyotype of FB has been previously published (Phelan et al, 1992). Arrows indicate the deleted chromosome. A schematic of chromosome 22 at the 850 band stage is presented below to indicate the bands that are present in a normal chromosome 22. The partial karyotypes were provided by Ikuko Teshima at the Hospital for Sick Children, Toronto (QM), Jerry Gorski at the University of Michigan, Ann Arbor (CH), the Genetic's Associates of Miami, Inc. (CB), A. Amir Al Saadl at the Medical College of Ohio, Toledo (NS), and David Ledbetter at the Baylor College of Medicine, Houston (AJ, JT).

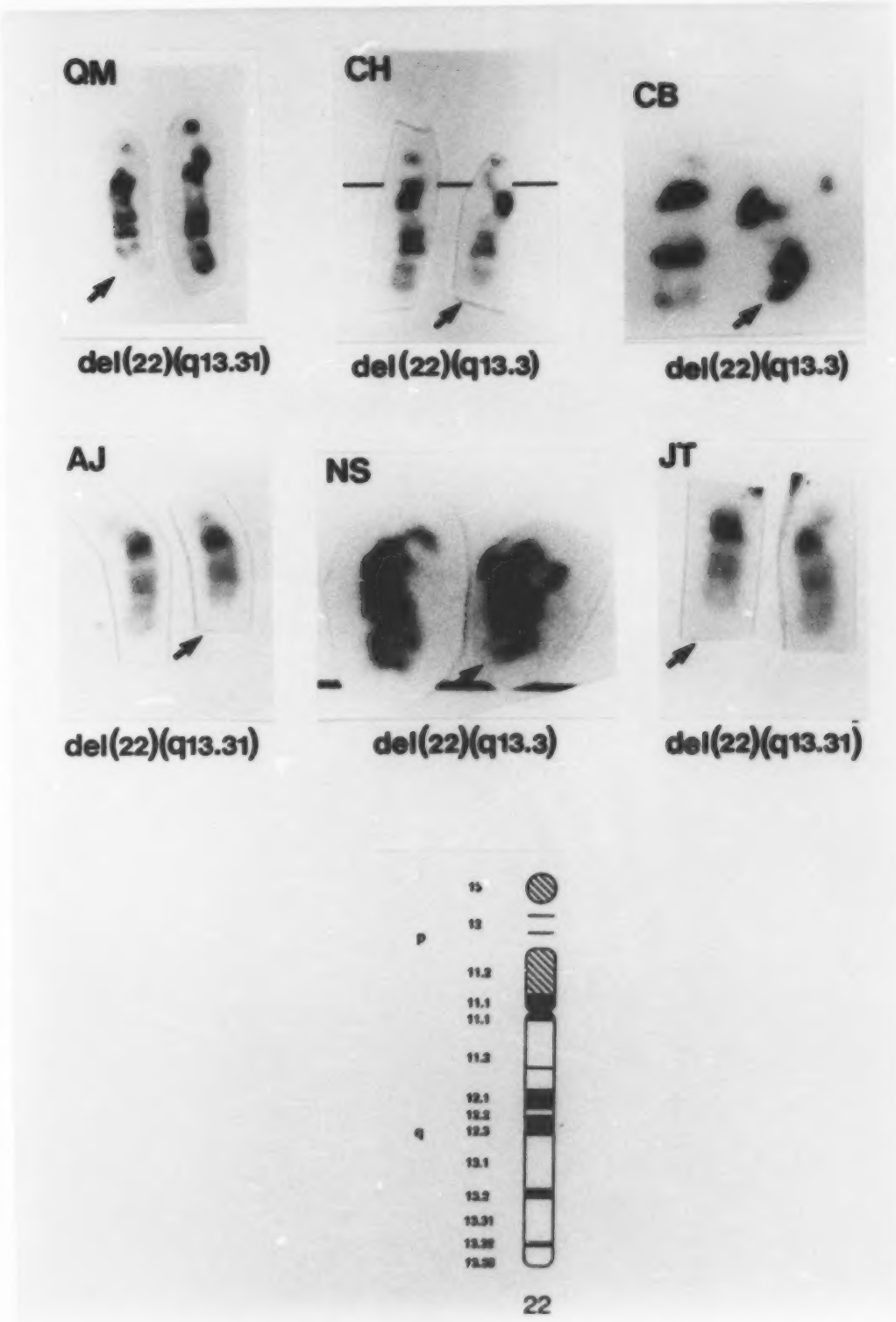

QM
del(22)(q13.31)
CH
del(22)(q13.3)
CB
del(22)(q13.3)
AJ
del(22)(q13.31)
NS
del(22)(q13.3)
JT
del(22)(q13.31)
p
q
22

Figure 3: Three examples of RFLP analysis in the del(22)(q13.3) patients

A. DNA was digested with TaqI and separated on a 0.8% agarose gel as described in the text. The resulting blot was hybridized to the D22S90 probe. The patients FB, QM, CH, NS, AJ and JT are all heterozygous for the polymorphism. FX (the mother of FB), patient CB and the normal control 3657 show only the 7.7 kb allele. 6420 is a ring 22 chromosome and BS is a possible chromosome 22 microdeletion. See text for complete details.

B. DNA was digested with EcoRV and separated on a 0.5% agarose gel as described in the text. The blot was hybridized to the D22S95 probe. FX, patients FB, CH, CB, and AJ and the parents of AJ (KJ and FJ) are heterozygous for the polymorphism. Patients QM, NS, JT, the mother of JT (MT), and the normal control 3657 show only the 15 kb allele. See text for complete details.

C. DNA from patients NS and JT plus the control 3657 were digested with HindIII and separated on a 0.8% gel. The resulting blot was hybridized to the D22S40 probe. JT is heterozygous for the polymorphism. 3657 and NS show only the 11.7 kb allele. See text for complete details.

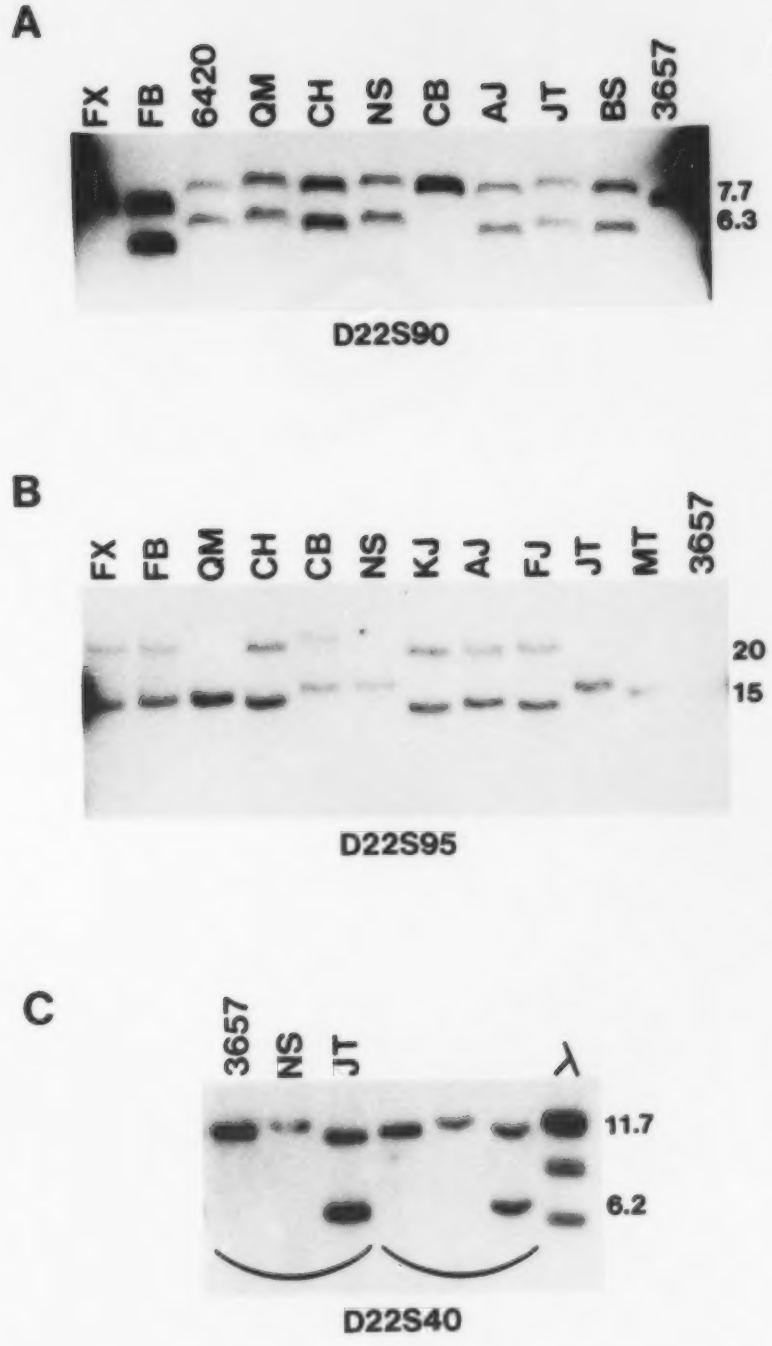

A
FX
FB
6420
QM
CH
NS
CB
AJ
JT
BS
3657
7.7
6.3
D22S90
B
FX
FB
QM
CH
CB
NS
KJ
AJ
FJ
JT
MT
3657
20
15
D22S95
C
3657
NS
JT
λ
11.7
6.2
D22S40

Table 4

Summary of RFLP Dosage Analysis

	Patient						
Locus	FB	QM	CH	CB	AJ	NS	JT
D22S102	2	2	-	2	-	-	nd
D22S90	2	2	2	-	2	2	2
D22S91	-	-	-	-	-	-	nd
PDGFB	-	-	-	2	-	-	nd
D22S92	-	-	-	-	-	-	nd
D22S95	2	-	2	2	2	-	-
D22S64	-	2	-	-	-	-	nd
D22S40	-	-	-	-	-	-	2
D22S94	-	-	-	-	1	-	-
D22S84	-	-	-	-	-	-	nd
D22S45	1*	-	-	-	1	-	nd
D22S55	1*	-	-	-	1	-	-
ARSA	-	-	-	-	-	-	nd

Probes are listed in order from most proximal to most distal (Dumanski et al. 1991).
2-indicates that the probe is heterozygous for the polymorphism and therefore is not deleted.
1-indicates that the probe is hemizygous, as determined by family studies.
1*-as reported by Phelan et al (1992).
(-) indicates that the probe is uninformative, showing the presence of only one allele in unknown dose.
nd- indicates that the probe was not determined.

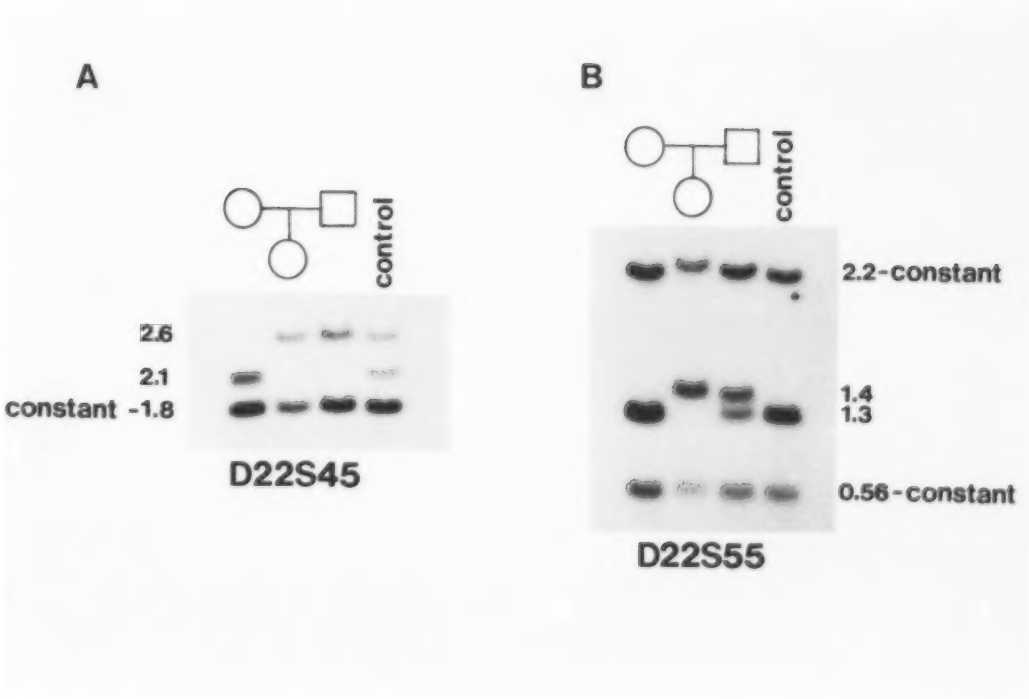
heterozygous for the polymorphism. The PDGFB and D22S64 probes were only informative in one case each. Patient CB and patient QM were heterozygous for the locus PDGFB and D22S64 respectively.

RFLP analysis was performed on patient AJ and her parents (KJ and FJ) to determine the origin of the deletion. Analysis using the D22S45 and D22S55 probes indicate that these loci are both deleted in patient AJ and that the deletion originated in the germ line of the mother (Figure 4). DNA from AJ and her parents was digested with PvuII and hybridized to the D22S45 probe (Figure 4A). AJ has inherited the 2.6 kb allele from her father (FJ) but has not inherited the 2.1 kb allele from her mother (KJ). Similar results were obtained with the D22S55 probe which recognizes a TaqI polymorphism (Figure 4B). AJ has inherited the 1.4 kb allele from her father but has not inherited the 1.3 allele from her mother. Locus D22S94 was also found to be in one copy in AJ due to a maternal deletion, using similar analysis. The remaining tested probes were uninformative in all cases.

Densitometric Analysis

Before densitometric analysis was undertaken several important controls were performed. The first consisted of a saturation control to ensure that the bands being read by the densitometer were in the linear range of the film. A dilution series, with DNA concentrations ranging from approximately 50 ug to 0.05 ug, was separated on a 0.8% gel by electrophoresis and transferred to nylon membrane as described above. The resulting blot was hybridized to D22S41 and the autoradiograph produced was scanned

Figure 4: Demonstration of a maternally derived deletion in AJ using D22S45 (A) and D22S55 (B) as probes. Genomic DNA was digested with PvuII or TaqI respectively and separated on a 0.8% gel. In each case AJ is shown to inherit one allele from her father but none from her mother.

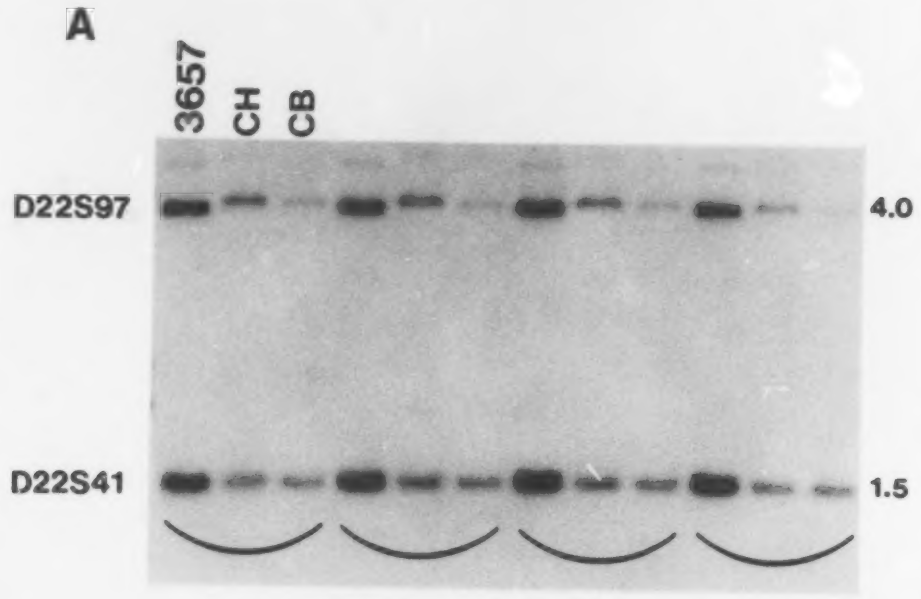

A
control
2.6
2.1
constant -1.8
D22S45
B
control
2.2-constant
1.4
1.3
0.56-constant
D22S55

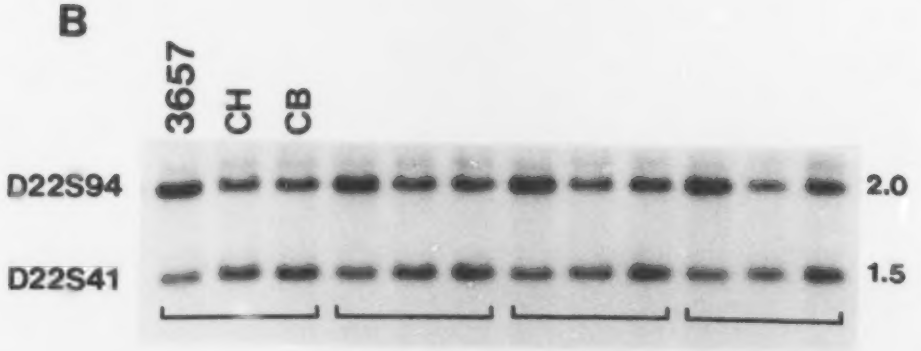
by the LKB Laser Densitometer. The resulting values were plotted on linear graph paper to determine the linear range of the film. Values of less than 0.200 and greater than 7.00 were considered to be outside the linear range and were not used when the statistical analysis was performed.

The second control was performed in order to ensure that 2:1 dosage differences could actually be distinguished by densitometry. DNA from a female patient and a male patient was separated by gel electrophoresis on a 0.8% agarose gel. The resulting blot was hybridized to a probe recognizing sequences on the X chromosome (DXS52) and a control probe located on chromosome 22q11 (N20). The X chromosome sequences should be in two copies in females and in one copy in males. Densitometry using the LKB Laser Densitometer was performed on the resulting autoradiograph. The statistical analysis (done as described in the Materials and Methods) demonstrated that the bands produced by the X probe were twice as dense in the female as in the male, indicating that it is possible to distinguish between 2 copies and 1 copy by densitometry.

Markers uninformative by RFLP analysis were used as probes for quantitative Southern blot analysis to determine the number of alleles present in each patient. An example of an autoradiograph used for densitometric dosage analysis is shown in Figure 5. DNA from patients CH, CB and a normal control, GM3657, were loaded in an alternating fashion such that four replicates were loaded per gel. The blot was initially hybridized to the D22S97 probe which shows a 4.0 kb HindIII fragment. The blot was simultaneously hybridized to the D22S41 probe, recognizing a 1.5 kb HindIII fragment. This probe

Figure 5: Autoradiographs used for densitometric analysis of the D22S97 (A) and D22S94 (B) probes. Four replicates (brackets) of DNA from a control, GM3657, and 2 patients, CH and CB, were separated by electrophoresis in a 0.8% agarose gel. The DNA was transferred to a nylon membrane and was hybridized simultaneously to the test D22S97 probe, which produces the 4.0 kb band and the control D22S41 probe (producing the 1.5 kb band) (A). Densitometric and statistical analysis demonstrated that CH was not deleted for D22S97, but that CB was deleted (see Table 5 for statistical analysis). The blot was then reprobed with another test probe, D22S94, producing the 2.0 kb band and the same control D22S41 probe. The band seen just above the 2.0 kb band is an additional faint band. Densitometric and statistical analysis demonstrated that both CH and CB were deleted for the D22S94 locus (statistical analysis provided in Table 5).


A
3657
CH
CB
D22S97
4.0
D22S41
1.5


B
3657
CH
CB
D22S94
2.0
D22S41
1.5

maps to the proximal region of 22q (Budarf et al, 1991) and therefore should be present in 2 copies in all samples, controlling for any lane-to-lane differences in DNA concentration (Figure 5A). Densitometric analysis indicated that the band corresponding to D22S97 was of equal intensity using the control as compared to CH, however the control was twice the intensity when compared to CB. This indicates that CH was not deleted at this locus but that CB was deleted (statistical analysis shown in Table 5). The blot was then hybridized to the D22S94 probe, which recognizes a 2.0 kb HindIII fragment, and with the D22S41 control probe (Figure 5B). The band corresponding to D22S94 is approximately twice as dense with the control DNA as compared to the patient DNAs, indicating the presence of a deletion in both CH and CB (statistics in Table 5).

The results of these analyses on each patient were combined with the RFLP data and are summarized in Table 6. For patients FB and AJ, the D22S92 and D22S95 probes were found to be in 2 copies (D22S95 by RFLP analysis with AJ), while the loci distal to and including D22S64 were all in one copy. This places the upper deletion breakpoint for FB and AJ between loci D22S95 and D22S64. In patients QM and JT, loci proximal to and including D22S40 were found to be in 2 copies, while the remaining distal loci were in 1 copy (D22S21 not done). This places the upper breakpoint between loci D22S40 and D22S97. In patient CH, the D22S64, D22S40 and D22S97 probes were found to be in 2 copies while the remaining distal loci were all in one copy. This places the upper breakpoint between loci D22S97 and D22S94. The most distal locus in patient CB that is in 2 copies is D22S64. The remaining more telomeric loci were in one

Table 5- Summary of statistical analysis performed on densitometry of autoradiographs with patients CH and CB hybridized to the D22S97 and D22S94 probes.

D22S97:

	3657	r	CH	r
1	0.721	8	0.720	7
2	0.685	6	0.645	5
3	0.635	4	0.598	3
4	0.522	2	0.481	1

total rank sum: 20 16

p-value: >0.5

conclusion: no significant difference ∴ CH not deleted

	3657	r	CB	r	3657	r	CB-D	r
1	0.721	8	0.350	4	0.721	8	0.700	7
2	0.685	7	0.280	2	0.685	6	0.560	3
3	0.635	6	0.337	3	0.635	4	0.674	5
4	0.522	5	0.102	1	0.522	2	0.204	1

total rank sum: 26 10 20 16

p-value: <0.01 >0.5

conclusion: significant difference; no significant difference when CB doubled ∴ CB deleted

D22S94:

	3657	r	CH	r	3657	r	CH-D	r
	2.05	8	0.841	1	2.05	4	1.68	1
	1.77	5	1.28	3	1.77	5	2.56	7
	1.90	6	1.41	4	1.90	6	2.82	8
	2.01	7	0.870	2	2.01	3	1.74	2

total rank sum: 26 10 18 18

p-value: <0.01 >0.5

conclusion: significant difference; no significant difference when CH doubled ∴ CH deleted

	3657	r	CB	r	3657	r	CB-D	r
	2.05	8	0.905	2	2.05	8	1.81	3
	1.77	5	0.864	1	1.77	2	1.73	1
	1.90	6	0.950	3	1.90	5	1.90	4
	2.01	7	0.972	4	2.01	7	1.94	6

total rank sum: 26 10 22 14

p-value: <0.01 0.10

conclusion: significant difference; no significant difference when CB doubled ∴ CB deleted

r = rank, D = values were doubled

p-value indicates the significance; if p-value>0.05, no significant difference is indicated

if p-value<0.05, a significant difference is indicated

Table 6
Summary of Densitometric and RFLP Dosage Analysis

	Patient						
Locus	FB	QM	CH	CB	AJ	NS	JT
D22S102	2	2	-	2·	-	-	-
D22S90	2·	2·	2·	-	2·	2·	2·
D22S91	-	-	2	2	2	-	-
PDGFB	-	-	-	2·	-	-	-
D22S92	2	2	2	2	2	2	2
D22S95	2	2	2·	2·	2·	1	2
D22S64	1	2	2	2	1	1	2
D22S40	1	2	2	1	1	1	2·
D22S97	1	1	2	1	-	1	1
D22S94	1	1	1	1	1·	1	1
D22S22	1	1	1	1	-	1	1
D22S82	1	1	1	1	1	1	1
D22S84	1	1	1	1	1	1	1
D22S45	1	1	1	1	1·	1	1
D22S21	1	-	1	1	-	-	-
D22S55	1	1	1	1	1·	-	-
ARSA	1	1	1	1	1	1	1

Probes are listed in order from most proximal to most distal (Dumanski et al. 1991).
2- indicates 2 copies of the DNA marker
1- indicates 1 copy of the DNA marker, consistent with deletion
·- information based solely on RFLP analysis
(-) indicates not determined

copy, placing the breakpoint between loci D22S64 and D22S40. In patient NS the D22S92 probe is in 2 copies while the remaining distal loci are all in 1 copy. This places the deletion breakpoint between loci D22S92 and D22S95. ARSA, the most distal probe known to date, is deleted in all of the patients. Therefore no lower breakpoint can be determined in any of the cases. A summary of the location of all the breakpoints is shown in Figure 6.

Confirmation of densitometric results in CH using somatic cell hybrids

A human/hamster somatic cell hybrid using CH cells as the human parent was produced by Dr. Marcia Budarf at the Philadelphia Children's Hospital. This hybrid contains the deleted chromosome 22 from CH as well as other human chromosomes, but it does not contain the normal chromosome 22. This hybrid was used to confirm the densitometric results obtained in patient CH. As Table 6 indicates, the most distal locus in 2 copies in CH was D22S97 and the most proximal locus in 1 copy was D22S94. These two loci were tested on the CH hybrid. The results are shown in Figure 7. DNA from a hamster cell line (RJK88), a hybrid containing chromosome 22 as the only human component (10888), the CH hybrid (CH), and a control normal human cell line (GM3657) were digested with HindIII and hybridized to the D22S97 probe (Figure 7A). As expected, there was no signal in the hamster control lane but a signal was detected in 10888, the CH hybrid and the total human control, indicating that D22S97 was indeed present in 2 copies in CH. The blot was then hybridized to the D22S94 probe (Figure 7B). Again, no signal was

Figure 6: A partial idiogram of chromosome 22q illustrating the loci detected by the DNA markers used in this study relative to each other with the recombination fractions (Θ) indicated (Dumanski et al, 1991). The extent of the deletion in each patient is indicated, as is the proposed critical region. The top of the bar was placed half way between the last locus that was in 2 copies and the first locus that was in one copy. The dots at the bottom of the bars indicate that the deletion extends distal to ARSA. The proximal breakpoints vary over a distance of approximately 13.8 cM.

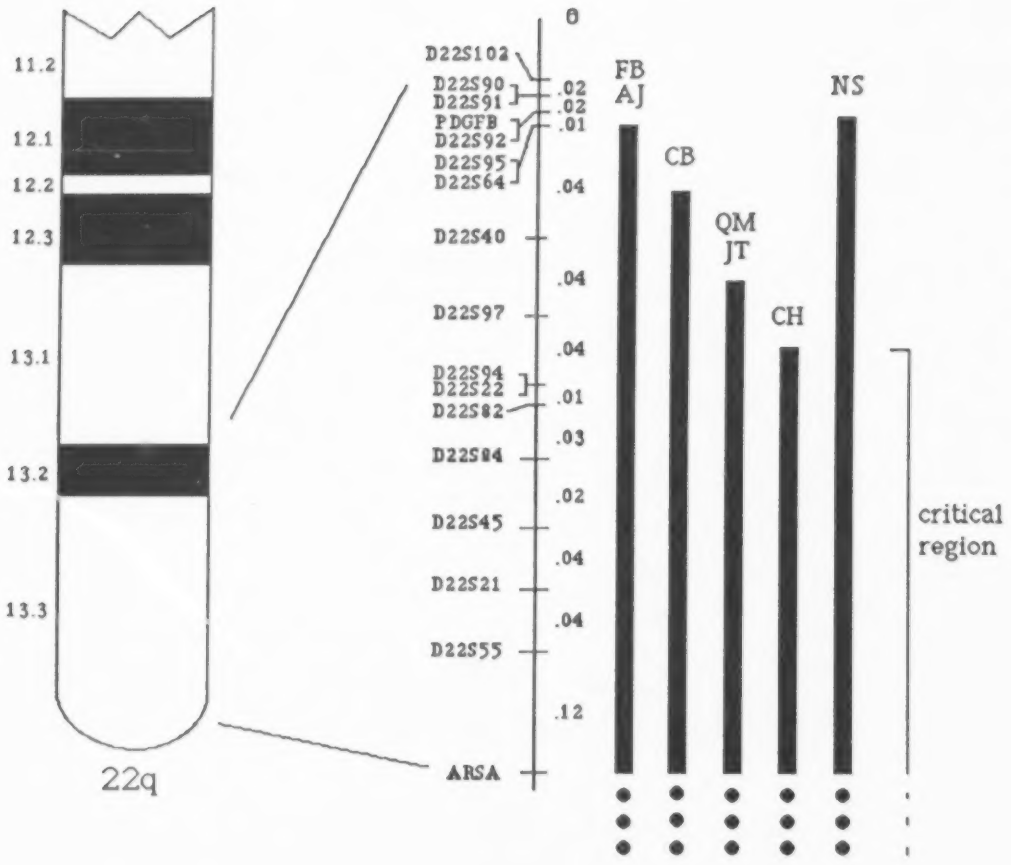

11.2
12.1
12.2
12.3
13.1
13.2
13.3
22q
0
D22S102
D22S90
D22S91
PDGFB
D22S92
D22S95
D22S64
D22S40
D22S97
D22S94
D22S22
D22S82
D22S84
D22S45
D22S21
D22S55
ARSA
.02
.02
.01
.04
.04
.04
.01
.03
.02
.04
.04
.12
FB
AJ
CB
QM
JT
CH
NS
critical
region

Figure 7: Confirmation of densitometric results in patient CH using the CH hybrid. DNA from a hamster cell line (RJK88), a human-hamster hybrid containing chromosome 22 as its only human component (10888), a human-hamster hybrid containing the deleted chromosome 22 from patient CH (CH), and a normal human cell line (3657) was digested with HindIII and separated on a 0.8% agarose gel. The resulting blot was hybridized to the D22S97 probe (A) and subsequently to the D22S94 probe (B). No signal is seen in either case in RJK88, as expected, since it contains no human component. A signal was present in 10888 and 3657 in both cases as expected since both of these cell lines contain at least one normal human chromosome 22. D22S97 produced a signal in the CH hybrid (A), indicating that this locus is not deleted in patient CH. However, no signal was produced by D22S94 in the CH hybrid (B), indicating that this locus is deleted in patient CH. These results confirm the results obtained by densitometric analysis.

RJK88
10888
CH
3657
A
D22S97
B
D22S94

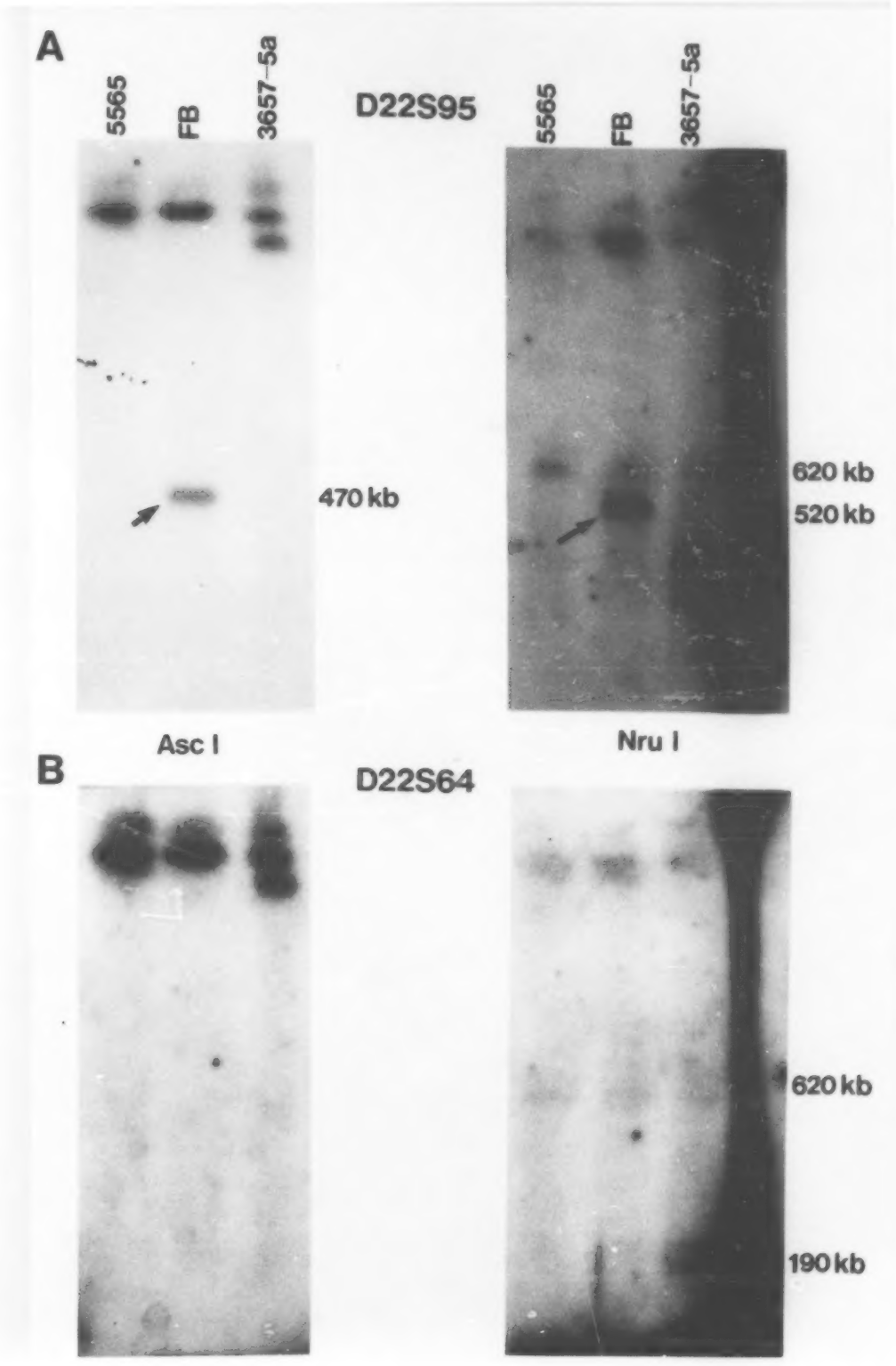
detected in the hamster control. A signal was seen in 10888, as expected since it contains an intact chromosome 22, and in the total human control. However, no signal was seen in the CH hybrid, indicating that this locus is deleted in CH. These results confirm the densitometric data for this patient.

Pulsed field gel electrophoresis

An attempt to visualize the deletion breakpoints by pulsed field gel electrophoresis was made for several patients. Emphasis was given to those patients in which the region of the upper breakpoint could be assigned to a small genetic distance with densitometric analysis. In both patients FB and AJ, dosage analysis indicated that the most distal locus not deleted was D22S95 and the most proximal locus deleted was D22S64. Genetic linkage analysis indicated that these two loci were inseperable by recombination (Dumanski et al, 1991), placing them less than 1000 kb from one another based on the fact that 1 cM approximately equals 1000 kb (Gelehrter and Collins, 1990). PFGE analysis of patient FB demonstrated the presence of possible rearrangement bands with two rare-cutting restriction enzymes (Figure 8).

Rare-cutting restriction enzymes such as NotI, NruI, and AscI cut DNA less frequently than other restriction enzymes because they contain 8 or 6 bp recognition sequences and the recognition sequences usually contain one or more CpG dinucleotides, which are underrepresented in the human genome (Jordan et al, 1988). In addition, CpG dinucleotides are often methylated at the cytosine, which prevents cleavage by enzymes that recognize these sequences

Figure 8: Study of the proximal FB deletion breakpoint by pulsed field gel electrophoresis. Agarose plugs containing DNA from a fibroblast control (GM5565), FB, and a 5-azacytidine lymphoblastoid control (GM3657-5a) were digested with AscI or NruI and separated in a 1.0% agarose gel by pulsed field gel electrophoresis under the conditions described in the Materials and Methods. The resulting blot was hybridized with the D22S95 probe, the locus directly proximal to the upper breakpoint of the deletion in FB. A novel AscI band of 470 kb and a novel NruI band of 520 kb appeared in FB which were not present in either of the controls (arrows) (A). The blot was then hybridized to the D22S64 probe, the locus immediately adjacent to D22S95 but distal to the breakpoint. Neither of the bands are present (B), as expected if the locus is deleted in this patient. Therefore the two novel bands represent the rearrangement breakpoint which is no more than 470 kb from D22S95.


A
5565
FB
3657-5a
D22S95
5565
FB
3657-5a
470 kb
620 kb
520 kb
Asc I
Nru I
B
D22S64
620 kb
190 kb

(Bird, 1986). 5-azacytidine demethylates CpG dinucleotides, and therefore it can uncover rare-cutting enzyme sites that are normally inaccessible to the enzyme (Dobkin et al, 1987). Since cells in culture sometimes show limited loss of CpG methylation, comparison to the 5-azacytidine control can prevent such a demethylation artifact from being mistaken for a rearrangement band (McDermid et al, manuscript in preparation).

Agarose plugs containing DNA from a fibroblast control (GM5565), FB, and a 5-azacytidine-treated lymphoblastoid control (GM3657-5a) were digested with AscI and NruI. The resulting blot was hybridized with the D22S95 probe, which is directly proximal to the upper breakpoint of the deletion in FB. A novel AscI band of 470 kb and a novel NruI band of 520 kb appeared in FB which were not present in either of the controls (Figure 8A). The blot was then reprobed with the D22S64 probe, which is immediately adjacent to the D22S95 probe but distal to the breakpoint. Neither of the novel bands are present (Figure 8B), as expected if the D22S64 probe is deleted in this patient. Thus, the two novel bands most likely identify the rearrangement breakpoint which is no more than 470 kb from the D22S95 probe, based on the size of the smallest novel band. The normal control lanes gave similar banding patterns when D22S95 and D2264 were compared, indicating that the two probes are on the same 620 kb NruI fragment and probably the same large AscI fragment.

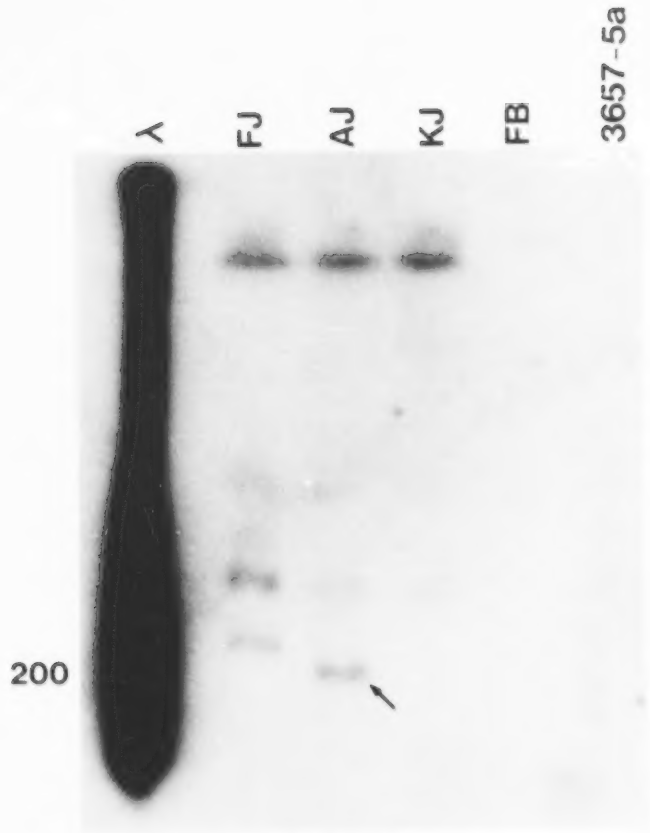
Similar analysis was undertaken with patient AJ. No novel bands were seen when AJ DNA was cut with AscI or NruI. However, preliminary analysis with AJ has demonstrated the presence of a

rearrangement band with the restriction enzyme BsiWI (Figure 9). Agarose plugs containing DNA from AJ and her parents (KJ and FJ), plus a 5-azacytidine lymphoblastoid control (GM3657-5a) were digested with BsiWI. The resulting blot was hybridized with the D22S95 probe. A novel band of approximately 200 kb was seen in AJ which was not present in either of her parents. Unfortunately the 5-azacytidine control did not work, so it is impossible to tell if the novel band is indeed a rearrangement band or if it is a methylation artifact. To compare the breakpoints in AJ and FB, DNA from FB was also separated on the gel, however this lane also failed to work. Previous analysis with FB DNA cut with BsiWI indicated that a similar novel band is not produced.

Yeast artificial chromosomes

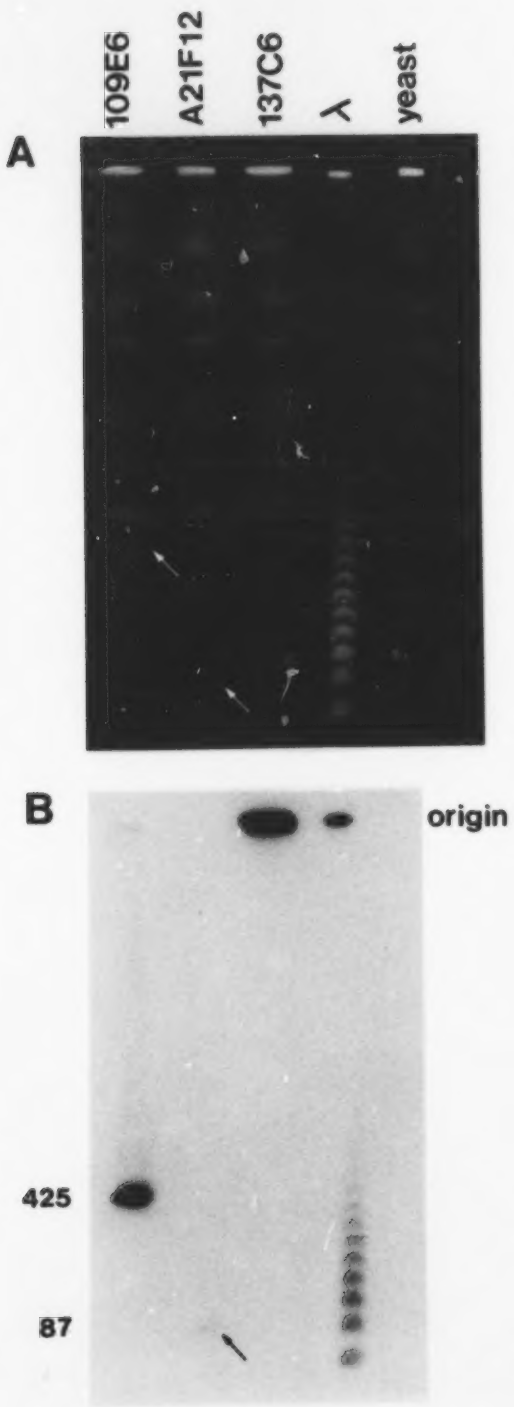
Three YACs containing D22S95 sequences were obtained from Dr. Ian Dunham at Guy's Hospital in London, England. These YACs were tested to see if they also contained the sequences that recognized D22S64. If so, we could conclude that the YAC would contain the deletion breakpoint of FB and AJ. First, the sizes of the YACs were determined. Agarose plugs containing DNA from each of the YACs were separated by pulsed field gel electrophoresis (Figure 10A). YACs 109E6 and A21F12 were visible among the other yeast chromosomes (arrows) and were determined to be 425 and 87 kb respectively. However, the third YAC, 137C6, was not visible. The resulting blot was probed with total human DNA in order to confirm that the YACs contained human DNA and to confirm the sizes of the YACs (Figure 10B). YAC 137C6 was found to be confined to the well,

Figure 9: Pulsed field gel electrophoresis with patient AJ. Agarose plugs containing DNA from AJ's father (FJ), AJ, AJ's mother (KJ), FB and a 5-azacytidine-treated lymphoblastoid control (3657-5a) were digested with BsiWI and separated by pulsed field gel electrophoresis under the conditions described in the Materials and Methods. A novel band of approximately 200 kb is seen in AJ which is not seen in either of her parents (arrow). The FB and the 3657-5a lanes did not work, making it impossible to tell if the novel band in AJ is a rearrangement band or merely a methylation artifact and to compare it to FB.



D22S95

Figure 10: Size determination of 3 YACs containing D22S95. Agarose plugs containing the YACs 109E6, A21F12, and 137C6 were separated by pulsed field gel electrophoresis in a 1.0% agarose gel under the conditions described in the Material and Methods (A). The YACs 109E6 and A21F12 were visible amongst the normal yeast chromosomes (arrows). However, the YAC 137C6 was not visible. The resulting blot was hybridized to total human DNA (3657) to confirm that the YACs contained human DNA and to confirm their size (B). Wild-type lambda labeled with 32PdCTP was also included in the hybridization solution to light up the lambda ladder. YAC 109E6 was determined to be 425 kb long and YAC A21F12 was determined to be 87 kb long. YAC 137C6 was found to be confined to the well, indicating that it may possibly be a circular YAC, and making its size impossible to determine without further manipulation. A lambda "ladder" (λ) and yeast chromosomes isolated from *Saccharomyces cerevisiae* (yeast) were used as standards to determine the sizes of the YACs.


A
109E6
A21F12
137C6
λ
yeast
B
origin
425
87

indicating that it may be circular and therefore unable to enter the gel under PFGE conditions.

The YACs containing D22S95 were then tested to see if they also contained the sequences corresponding to D22S64, the locus adjacent to D22S95, but within the deletion. Since the D22S64 probe shows unexplained cross-hybridization to yeast sequences, two cosmids, N32H10 and N72F5, containing the D22S64 locus were obtained to produce a new hybridization probe. These cosmids were cut with SstI, which does not cut within the YAC vector. Fragments were isolated on a low-melt agarose gel and tested to see if they were unique. Five unique fragments, N72F51.2, N72F53.2, N72F53.5, N32H101.0, and N32H101.8, were then tested to see if they also recognized the YACs containing D22S95. All of them were negative, indicating that none of the YACs contained both loci D22S95 and D22S64.

Localization of genes and potential genes with the CH hybrid

As indicated by the dosage analysis results, patient CH has the most distal deletion, extending from D22S97 to a region distal to ARSA. Therefore the CH hybrid is a useful tool to explore whether certain probes or genes roughly localized to 22q13.3 are located within the smallest region of overlap (critical region) shared by the seven deletion patients. Two genes encoding S-lac lectins, LGAlS1 and LGALS2, act as lactose binding proteins and have been localized to chromosome 22q12-q13 by somatic cell hybrids and *in situ* hybridization (Mehrabian et al, 1993). Probes identifying these

genes were tested on the CH hybrid to see whether or not they were located within the critical region. As shown in Figure 11, LGALS2 is present in the CH hybrid, indicating that it is not located within the critical region. LGALS1 was also found to be present in the CH hybrid indicating that this gene is also not located within the critical region.

A number of sequences, possibly directly adjacent to genes were also localized. A set of probes, designated "EN", were previously isolated from a bacteriophage library composed of size-selected NotI/Sau3AI DNA fragments from a human/hamster hybrid cell line containing chromosome 22 as its major component (McDermid et al, 1989). It has been estimated that NotI sites occur every 800-1200 kb in the human genome (Barlow and Lehrach, 1987), however most NotI sites appear to be concentrated in CpG islands. CpG islands are 1-2 kb sized regions of DNA that are rich in unmethylated CG sequences. These regions contain abundant infrequently cutting enzyme sites, such as NotI (Brown and Bird, 1986). These sites are usually cleavable in genomic DNA due to the lack of methylation. All of the EN clones contain a NotI site, indicating that these probes may be located within CpG islands, which are often found at the 5' ends of genes (Bird, 1986). Thus, these probes may locate possible sites of genes. Several EN probes had previously been localized to the distal portion of 22q using a somatic cell hybrid from a cell line with a Ewing's sarcoma-associated t(11;22)(q23-24;q11.2-q12) translocation (unpublished data). These EN clones were tested on the CH hybrid to determine if any could be further localized to the 22q13.3 critical region. The probes and the results are summarized in Table 7 and two examples are shown in

Figure 11: Testing the gene LGALS2 and "EN" clones on the CH hybrid. DNA from a hamster cell line, RJK88, a human/hamster hybrid containing chromosome 22 as its only human component, 10888, the CH hybrid and a normal human cell line, 3657, were digested with HindIII and separated on a 0.8% gel by electrophoresis. The resulting blot was hybridized to LGALS2. A signal is present in the CH hybrid (CH) indicating that LGALS2 is not within the shortest region of overlap defined by the seven deletion patients. The blot was subsequently hybridized to EN20, which produces a 1.5 kb signal in the CH hybrid, indicating that this clone is also not within the critical region. The blot was then hybridized to EN11. A 25 kb signal was only produced in 10888 and 3657, the two positive control lanes. No signal was seen in the CH hybrid, indicating that this clone is present in the critical region.

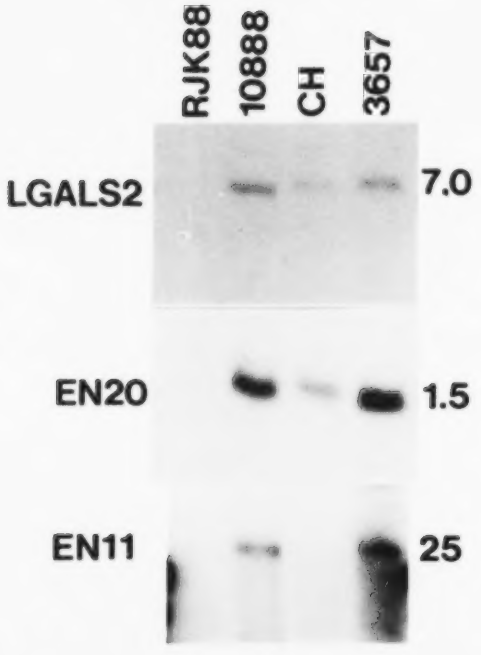

RJK88
10888
CH
3657
LGALS2
7.0
EN20
1.5
EN11
25

Table 7- Results of EN probes tested on the CH hybrid

clones below Ewing sarcoma breakpoint	fragment	below CH?
EN8	1.6 HE	no
EN9	2.0 HE	yes
EN11	1.2 HE	yes
EN20	1.3 HE	no
EN22	1.7 S	no
EN26	1.7 HE	no
EN30	1.9 HE	no
EN35	0.6 HE	no
EN38	1.2 HE	no
EN39	0.9 S	ND
EN45	0.8 HE	no
EN47	2.3 HE	ND
EN60	0.4 HE	no
EN61	2.5 S	ND
EN62	1.2 S	ND
EN65	1.5 HE	ND
EN66	1.6 HE	ND
EN81	1.1 HE	ND
EN84	2.0 HE	yes

Fragment sizes in kb followed by enzymes used
H-HindIII, E-EcoRI, S-SacI
ND-not determined

Figure 11. Three probes, EN9, EN11, and EN84 were not present in the CH hybrid, indicating that they are located in the deleted region.

Fluorescent *in situ* hybridization

Fluorescent *in situ* hybridization (FISH) was performed on most of the deletion patients to confirm the status of ARSA. ARSA is the most distal locus on chromosome 22q known to date, thus the status of ARSA is of particular importance. If ARSA is in 2 copies in the 22q13.3 deletion patients, the deletion can be confirmed as an interstitial deletion. However if ARSA is deleted in the patients, it is currently impossible to determine if the deletion is interstitial or terminal. As shown in Table 6, densitometry indicated that ARSA was deleted in all of the 22q13.3 deletion patients. However the probe recognizing ARSA was very difficult to work with, thus it was important to confirm if ARSA was actually deleted. A cosmid containing sequences recognizing ARSA, designated 73A6 was obtained from James Troffater at the Harvard Medical School in order to perform the FISH analysis.

The results of the FISH analysis performed with ARSA are presented in Table 8. Since the chromosomes are not banded, the chromosome 22s were identified by including a cosmid in the hybridization to give a second signal at 22q11 at locus D22S75. A signal corresponding to D22S75 was seen on all four chromatids in 95% of the metaphase spreads examined. In the remaining 5%, a signal was seen on three of the four chromatids. Therefore, both chromosome 22 homologues were identified in every metaphase spread examined. A signal was seen for ARSA in 76%-91% of the

Table 8-Frequency of signals for loci D22S75 and ARSA using FISH analysis on the chromosome 22q13.3 deletion patients

Cell line and probe	Both 22s	One 22	Neither 22	# scored
QM				
D22S75	26 (100%)	0	0	26
ARSA	0	22 (85%)	4 (15%)	26
CH				
D22S75	16 (100%)	0	0	16
ARSA	0	14 (87.5%)	2 (12.5%)	16
CB				
D22S75	20 (100%)	0	0	20
ARSA	0	16 (80%)	4 (20%)	20
AJ				
D22S75	25 (100%)	0	0	25
ARSA	0	21 (84%)	4 (16%)	25
NS				
D22S75	21 (100%)	0	0	21
ARSA	0	16 (76%)	5 (21%)	21
JT				
D22S75	22 (100%)	0	0	22
ARSA	0	20 (91%)	2 (9%)	22

metaphase spreads examined, depending on the patient. None of the metaphase spreads examined had a signal for ARSA on both of its homologues, indicating that ARSA was deleted in one of the chromosome 22s in each case. Figure 12 shows sample metaphase spreads examined with FISH in CB and JT. These results confirm that ARSA is deleted in all of the 22q13.3 deletion patients.

Figure 12: Fluorescent in situ hybridization using ARSA as a probe. Metaphase spreads from patient CB (A) and JT (B) were simultaneously hybridized to D22S75, a control probe to identify both chromosome 22s, and the test probe, ARSA. Arrows indicate the chromosome 22s. In each case one chromosome 22 has 2 signals, indicating the presence of both D22S75 (bright) and ARSA (faint) sequences. The other chromosome 22 has only one signal, that of D22S75, indicating that ARSA is deleted.

A

B

DISCUSSION

Clinical analysis

This study describes seven patients displaying similar visible deletions of 22q13.3. These patients share a common phenotype that includes developmental delay, hypotonia, normal or accelerated growth, relatively severe expressive speech delays, and minor facial dysmorphic features. The five previously published cases also displayed some of these characteristics (the Phelan et al (1992) patient is included in this study). The patients described by Romain et al (1990) and Narahara et al (1992) both demonstrated developmental delay and hypotonia, while the patient described by Kirschenbaum et al (1988) showed developmental delay.

The dysmorphic features are difficult to compare because there is a lack of detail in the published reports and the previously published cases were specifically being compared to ring(22) cases. The observation that all of our patients display normal or accelerated growth was also seen in some of the previously published cases, with one case over the 97th percentile in height (Romain et al. 1990), one with macrocephaly (Herman et al. 1988), and one with normal growth (Narahara et al. 1992). This is unusual since most deletion syndromes as well as most duplication syndromes described are noted for growth retardation (Epstein et al. 1986). According to Schinzel (1988), the only autosomal chromosomal aberrations that demonstrate normal growth are cat eye syndrome, del(9)(pter-p21) and dup(13)(q12-qter). One of the previously published cases was

actually microcephalic (Watt et al. 1985), however this patient had a much larger deletion, extending from 22q12-qter.

All of the patients in this study demonstrated severe delays or complete absence of expressive speech. This was also seen in the previously published cases. Two cases (Watt et al, 1985; Romain et al, 1990) had no speech while one case (Kirschenbaum et al, 1988) demonstrated speech delays. In the remaining previously published cases (Herman et al, 1985; Narahara et al, 1992) the patients were too young for speech development to be ascertained. Delays or absence in expressive speech is not very common in aneuploidy syndromes. In Smith-Magenis syndrome (Greenberg et al, 1991) and trisomy 9p syndrome which involves a duplication of chromosome 9(pter-p12 or p13) (Jones, 1988), speech is usually severely delayed. In Angelman syndrome speech is completely absent (Jones, 1988). All of the patients in this study were under the age of 5 at their last medical exam, therefore their phenotype will likely become clearer over time.

Patients with large scale autosomal chromosome aberrations are usually characterized by four major findings: mental deficiency, a specific pattern of minor dysmorphic features, major malformations, and pre- and postnatal growth retardation (Schinzel, 1988). The del (22)(q13) patients presented in this study are unusual in that they do not follow this pattern. They do demonstrate a somewhat similar pattern of minor dysmorphic features, however they show no major malformations, and no growth retardation. In fact, all of the patients demonstrate normal or accelerated growth. Their mental deficiency has not been evaluated to date, mainly because they are quite young

(all under 5 years) and mental ability is difficult to ascertain at a young age. They demonstrate severe delays in expressive speech, however this does not necessarily reflect their mental ability. Their delays may be due to the hypotonicity displayed by all of the patients. Thus, it seems that this syndrome is relatively mild for an aneuploid syndrome in which a considerable amount of genetic material (>25 cM) has been lost. Again, because the patients are young it is difficult to predict what the adult phenotype will be.

Dosage analysis

To determine the extent of the chromosome 22 deletion in each patient, dosage analysis was performed using probes from the most complete linkage map of chromosome 22 published to date (Dumanski et al, 1991). As summarized in Figure 6, the upper breakpoints of the deletions vary over approximately 13.8 cM, between loci D22S92 and D22S94. Some of the breakpoints, however, are clustered. The breakpoints of FB and AJ lie between D22S95 and D22S64, with D22S95 in 2 copies and D22S64 in 1 copy. This refines the order of these loci, placing D22S95 proximal to D22S64. Since D22S95 and D22S64 are not genetically separable (Dumanski et al. 1991), this suggests that the two loci are approximately 1000 kb apart (Gelehrter and Collins. 1990).

The proximal breakpoints of both QM and JT lie within the same region between loci D22S40 and D22S97, a region spanning 4.4 cM. NS has the most proximal deletion, extending from the region between the D22S92 and D22S95 probes to below ARSA. CH has the most distal deletion, stretching from the region between the D22S97

and D22S94 probes to below ARSA. Even though NS has the biggest deletion he is the least dysmorphic looking (with only dysplastic ears) of the seven patients in this study. He was one of the earliest walkers, indicating that he was the least developmentally delayed. NS did have a ventricular septal defect but this was likely unrelated to the deletion. On the other hand, although CH has the smallest deletion, she is one of the most seriously affected patients. She has several dysmorphic features including dolicocephaly, epicanthal fold, dysplastic ears and downslanting palpebral fissures. In addition, CH was disproportionately large with macrocephaly and tall stature. Thus, it appears that the size of the deletion does not correlate with the severity of the phenotype in this syndrome. This is also seen in other deletion syndromes such as Wolf-Hirschhorn syndrome (Gandelman et al, 1992) and Angelman syndrome (Knoll et al, 1993) in which the size of the deletion does not seem to correlate with increased severity. This may indicate that genes between the NS and CH breakpoints are not susceptible to dosage effects or such effects are subtle. However, there are deletion syndromes in which the severity of the phenotype does correlate to the size of the deletion. An example is the $18q^-$ syndrome in which the deletions extend from 18q21.3 or 18q22.2 to qter (Kline et al, 1993). Patients with the larger deletion extending from 18q21.3-qter generally have a more severe phenotype compared to those with the smaller 18q22.2-qter deletion (Kline et al, 1993).

There is a great deal of variability in the phenotype of ring (22) cases. However, these patients tend to be more severely affected than the deletion 22q13.3 patients. The breakpoint of the

ring (22) chromosomes are thought to occur in 22q13, the same region as the 22q13.3 deletion breakpoints. This is supported by a preliminary study on one ring (22) case in which the breakpoint was localized to the same region as the del(22)(q13.3) patients (unpublished data). It was demonstrated in this study that the great variation in deletion size seen in the 22q13.3 deletion patients does not correlate to the severity of the phenotype. Thus, it is likely that the increased severity seen in the ring (22) cases is due to the general instability of ring chromosomes, rather than to the actual amount of DNA deleted.

The probe that recognizes ARSA is the most distal probe mapped to date and is deleted in all cases. It is unknown how far ARSA is from the telomere, therefore it is difficult to determine the exact size of the smallest region of overlap (critical region). However it can be defined as extending from D22S94 to a region distal to ARSA, a distance of greater than 25.5 cM (Figure 6).

RFLP analysis performed on AJ and her parents demonstrated that her deletion was maternally inherited. It was previously reported that the deletion in FB was paternally inherited (Phelan et al. 1992). This suggests that imprinting does not play a role in this phenotype as it does in Prader-Willi syndrome and Angelman syndrome (Knoll et al, 1989). There is also no evidence for imprinting in cat eye syndrome (Alan Mears, pers. comm.) or DiGeorge syndrome (Driscoll et al, 1992a), indicating that chromosome 22 likely does not show any imprinting effects.

Pulsed field gel electrophoresis and yeast artificial chromosomes:

The preliminary results with pulsed field gel electrophoresis of FB and AJ DNA indicate that DNA rearrangement bands can be seen. The proximal breakpoint of FB was determined to be no more than 470 kb from the D22S95 probe, since this was the size of the smallest rearrangement band. Preliminary results with AJ suggest that her proximal breakpoint may be no more than 200 kb from the D22S95 probe, however this result needs to be confirmed with further pulsed field analysis. According to the pulsed field results obtained to date, AJ does not display the same rearrangement bands that FB does, indicating that the breakpoints in FB and AJ are distinct. This is the initial step towards cloning the breakpoints of these deletions. Several YACs containing D22S95 sequences were obtained and tested to see if they also contained D22S64 sequences. If this was found to be the case it could be concluded that the YAC also contained the breakpoint of FB and AJ. Unfortunately, none of the YACs tested contained both D22S95 and D22S64 sequences. This does not mean that the YACs don't contain the deletion breakpoint for FB and AJ. In order to determine this for certain, YAC end recovery would have to be performed. This end clone could then be used to test if it is deleted by FISH or densitometry.

When attempts were made to separate the YACs by pulsed field gel electrophoresis, one YAC, 137C6, was found to be confined to the well. In order to determine the actual size of the YAC it would have to linearized with an enzyme that cut only once.

Cloning the deletion breakpoints will be important in determining the nature and distal limit of these deletions. Wilkie et al (1990) described a family segregating an α-thalessemia mutation associated with terminal truncation of the short arm of chromosome 16, in which the individuals carrying this mutation are of normal intellect. It was shown by sequencing that the chromosomal break was stabilized by the addition of the human telomeric repeat, $(TTAGGG)_n$, directly to the site of the break (Wilkie et al. 1990). An α-thalassemia/mental retardation syndrome patient with a *de novo* terminal deletion of 16p13.3 was also shown to have the telomeric sequence added to the site of the break (Lamb et al, 1993). This suggests that the chromosomal break has been stabilized *in vivo* by the direct addition of the telomeric sequence. When the breakpoints of these two cases were compared, no extensive sequence homology was found. However, both breakpoints show minimal complementarity (3-4 bp) to the proposed RNA template of human telomerase at the site where telomere repeats have been added (Lamb et al, 1993). Studies suggest that telomere synthesis can occur when only minimal complementarity to the RNA template of telomerase is present at the 3' terminus (Morin, 1991). Thus, a short 3' overhang at the junction site of either one of these breakpoints may be sufficient to prime telomere synthesis. This is the only human example of a telomere being added to the end of a deletion. The cloning of the FB and AJ breakpoints will allow us to determine if the deletions in these patients are truly terminal with only the telomeric repeat added, or if they are actually interstitial deletions.

We have recently received a cosmid clone that contains subtelomeric and telomeric sequences. This clone is specific to chromosome 22 by FISH analysis but also hybridizes to an oligonucleotide made up of the human telomeric repeat (Xie, pers. comm.). This clone provides a way to investigate whether the deletions in all of the patients in this study are truly terminal. If this probe is in one copy (by FISH or densitometry) in the del(22)(q13.3) patients then the deletions can be considered truly terminal and it would be like the α-thalessemia case.

Extensive YAC mapping of chromosome 22 is currently underway in several centers. This will provide new probes in the region between ARSA and the telomere. If the subtelomeric probe is found to be in two copies, indicating the deletion is interstitial, the new probes will allow the determination of the lower breakpoints by FISH or densitometry. Once the lower breakpoints have been determined in all the deletion patients, refinement of the critical region will be possible.

Localization of genes and potential genes

There have been several reports of genes that map to 22q13. The SSTR3 gene encodes a human somatostatin receptor that has been mapped to 22q13.1 by fluorescent *in situ* hybridization (Yamada et al, 1993). Two genes encoding S-lac lectins, LGALS1 and LGALS2, act as lactose binding proteins and both have been localized to chromosome 22q13 by somatic cell hybrids and *in situ* hybridization (Mehrabian et al, 1993). The human platelet-derived endothelial cell growth factor (ECGF1) has been shown to promote

angiogenesis *in vivo* and to stimulate the *in vitro* growth of endothelial cells. The ECGF1 gene has been localized to chromosome 22q13 by a panel of somatic cell hybrids and has been determined to map distal to PDGFB (Stenman et al, 1992). The CYP2D gene, which codes for an enzyme involved in the metabolism of exogenous substrates including many neuronally acting drugs, has been mapped to chromosome 22q13.1 by *in situ* hybridization. However, linkage studies gave a lod score of 8.12 (Θ=0) between the locus D22S64 and CYP2D6 and of 6.92 (Θ=0) between the locus D22S95 and CYP2D6 (Gough et al, 1993), which would put it outside the critical region. Finally, the α-N-acetylgalactosaminidase (NAGA) gene, deficient in Schindler disease, has been localized to chromosome 22q13-qter and encodes a lysosomal glycohydrolase that cleaves α-N-acetylgalactosaminyl moieties into glycoconjugates (Wang and Desnick, 1991).

The LGALS1 and LGALS2 genes were tested on the human/hamster hybrid containing the deleted chromosome of CH. Both of these genes were present in CH's deleted chromosome, indicating that they are not located within the critical region. Attempts are being made to obtain the other genes localized to 22q13 to see if any map within the critical region and possibly play a role in the phenotype of this syndrome. The genes described above that code for enzymes are not likely candidates to be involved in the development of this syndrome. A deletion will effect the dose of the gene product, which will likely not affect the action of an enzyme. Currently, ARSA is the only known gene in the area and it is not susceptible to dosage effects (Gieselmann, 1991).

The set of probes designated "EN" identify sequences that potentially lie adjacent to genes. All of the EN clones contain a NotI site, indicating that these probes may be located within CpG islands, which are often at the 5' ends of genes (Bird, 1986). A NotI junction clone from the same library (N41) detects a cDNA clone from a human fetal liver library (Budarf et al, 1992). Sequence analysis of the cDNA clone indicated that it was a gene that had not been previously identified. Northern blots were performed and a 4.5 kb transcript was detected in human heart, placenta, brain and liver. In addition, the clone cross-hybridizes with mouse genomic DNA suggesting evolutionary conservation. Another probe, N15, also adjacent to a NotI site, hybridizes to specific hamster and mouse bands, indicating the presence of a conserved region and possibly a gene (McDermid et al, 1989). Three EN clones; EN9, EN11, and EN84, were all found to be within the critical region when tested on the CH hybrid. These probes can now be used to look for primate or rodent cross-hybridization. If cross-hybridization is indicated, these probes can then be used to screen a human cDNA library in order to determine if they identify novel genes that may be involved in the phenotype of this syndrome.

The experience with other deletion syndromes such as DiGeorge syndrome (Driscoll et al, 1992a), Prader-Willi syndrome (Robinson et al, 1991), Angelman syndrome (Hamabe et al, 1991), Smith-Magenis syndrome (Greenberg et al, 1991), the WAGR locus (Fantes et al, 1992), Miller-Dieker syndrome (vanTuinen et al, 1988), and Wolf-Hirschhorn syndrome (Gandelman et al, 1992) indicates that microdeletions associated with the described phenotype in 22q13.3

could exist. Such microdeletions could be useful in further delineating the region involved in expression of the phenotype. We are currently searching for cases who fit the del(22)(q13.3) phenotype but have normal high-resolution G-banded analysis of chromosome 22. These cases can then be tested with probes in the critical region to see if any are deleted.

This study has led to the delineation of a new syndrome that has been defined by clinical, cytogenetic and molecular approaches. It has also provided the basis for further research. The directions that can now be followed include the search for microdeletions to help refine the critical region involved in this syndrome, the search for genes that contribute to the development of the phenotype and may provide a better understanding of human development in general, and the cloning of the breakpoints which will lead to an understanding of the mechanism of occurence of these deletions. This research has also provided some comfort to the families of the children involved in this study.

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